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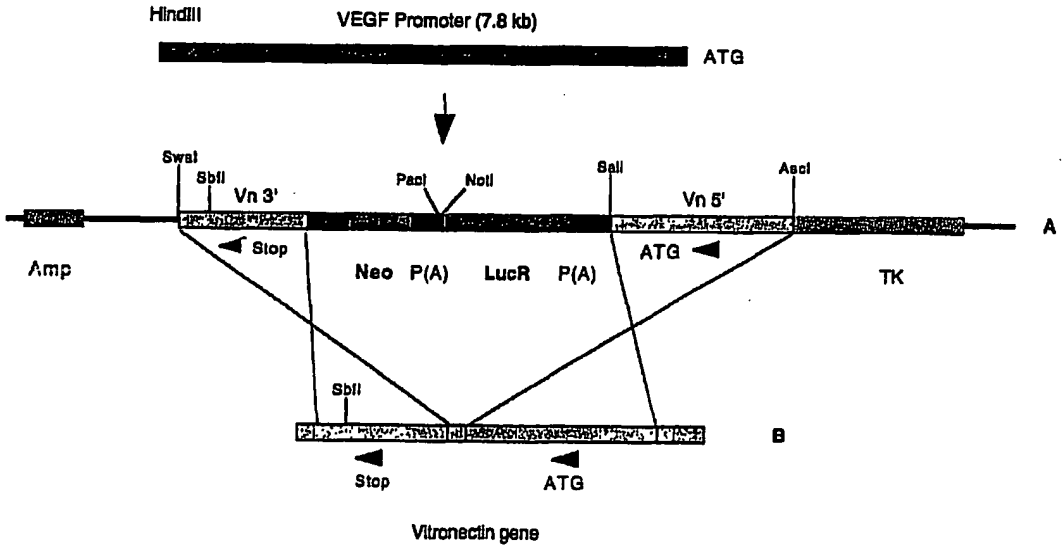
(54) Title: TARGETING CONSTRUCTS AND TRANSGENIC ANIMALS PRODUCED THEREWITH



(57) Abstract: The present invention teaches targeting constructs and methods of use thereof for creating transgenic animals in which at least one single-copy, non-essential gene is replaced with a reporter expression cassette, for example, a luciferase gene operably linked to a promoter heterologous to the single-copy, non-essential gene. Thus, the present invention provides novel methods and vector constructs useful for the generation of transgenic animals. The invention further includes methods of using these animals.

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TARGETING CONSTRUCTS AND TRANSGENIC ANIMALS PRODUCED THEREWITH

TECHNICAL FIELD

This invention is in the field of molecular biology and medicine. More specifically, it relates to novel vector constructs and methods of use thereof for introducing heterologous polynucleotides into a host cell. Further, the invention relates to vector constructs and methods of use thereof to generate transgenic organisms, particularly transgenic mice.

BACKGROUND

In recent years, mouse geneticists have succeeded in creating transgenic animals by manipulating the genes of developing embryos and introducing foreign genes into these embryos. Once these genes have integrated into the genome of the recipient embryo, the resulting embryos or adult animals can be analyzed to determine the function of the gene.

Traditionally, transgenic mice have been generated through DNA microinjection approach. Such an approach leads to the creation of "founder" mice having at least one copy of the transgene randomly integrated into the genome. Because neither the copy number nor the integration sites can be controled, transgenic mice generated by this method are genetically different from each other. The expression of transgenes in such transgenic mice are not uniform because of, for example, the difference in copy numbers of the transgene. Furthermore, chromosomal location of the transgene often affects the expression level of the transgene. For most of *in vivo* studies, particularly ones where it is desirable to compare levels of gene expression across different animals, it is important to have the mice with little or no genetic variation (i.e., isogenic mice) in order to reduce the systematic error.

One way of controlling copy number and the chromosomal integration site is by using targeting constructs to create transgenic animals. U.S. Patent Nos. 5,464,764 and 5,487,992 describe this type of transgenic animal in which a gene of interest is deleted or mutated sufficiently to disrupt its function. These "knock-out" animals are made by taking advantage of the phenomena of homologous recombination. (*See, also* U.S.

Patent Nos. 5,631,153 and 5,627,059). Using the "knock-out" approach, when the vector is introduced into the embryonic stem cell, a sequence becomes integrated into a target gene in the genome via homologous recombination. The integration disrupts the function of the target gene allows for examination of the phenotype resulting from the disruption of the gene. Neither of the above methods, however, provide a predictable approach for the generation of gene expression reporter transgenic animals which can be used for quantitative comparisons of gene expression across different animals transfected with the same construct, or for quantitative comparisons of the relative levels of expression of two or more different genes.

The present invention solves this and other problems by providing transgenic animals in which at least one single-copy, non-essential gene is replaced with a reporter expression cassette, for example, a luciferase gene operably linked to a heterologous promoter. Thus, the present invention provides novel methods and vector constructs useful for the generation of transgenic animals. The invention further includes methods of using these animals.

SUMMARY OF THE INVENTION

The transgenic animals described herein are useful, for example, when studying *in vivo* regulation of selected genes. Also described herein are methods of generating populations of substantially isogenic transgenic animals, as well as, vectors useful in these methods.

Accordingly, in one embodiment, the subject invention is directed to a transgenic, non-human mammal, for example, a rodent such as a mouse. The mammal comprises at least one single-copy, non-essential gene in its genome, wherein (i) at least a portion of at least one single-copy, non-essential gene is replaced by polynucleotide sequences heterologous to the gene, and (ii) the polynucleotide sequences comprise a first expression cassette which has been introduced into the mammal or an ancestor of the mammal, at an embryonic stage. The first expression cassette typically comprises a first selectable marker, a first transcriptional promoter element heterologous to the gene, and light-generating protein coding sequences. The light-generating protein coding sequences are operably linked to the promoter element.

The single-copy, non-essential gene may be selected, for example, from the group consisting of vitronectin, *fosB*, and galactin 3 and the first selectable marker may be selected from the group consisting of neomycin phosphotransferase II, xanthine-guanine phosphoribosyltransferase, hygromycin-B-phosphotransferase, chloramphenicol acetyltransferase, and adeninephosphoribosyl transferase.

In alternative embodiments, the first transcriptional promoter element is an inducible promoter, a repressible promoter, or a constitutive promoter, and may be selected from the group consisting of VEGF, VEGFR, and TIE2.

In additional embodiments, the transgenic, non-human mammal described above comprises a second single-copy, non-essential gene in its genome, wherein (i) at least a portion of the second single-copy, non-essential gene is replaced by polynucleotide sequences heterologous to the second gene, and (ii) the polynucleotide sequences comprise a second expression cassette which has been introduced into the mammal or an ancestor of the mammal, at an embryonic stage. The second expression cassette typcially comprises a second selectable marker, a second transcriptional promoter element heterologous to the second gene, and light generating protein coding sequences. The light generating protein coding sequences are operably linked to the promoter element.

The first and second transcriptional promoter elements and selectable markers may be the same or different and the light generating protein in the first expression cassette can produce a different color of light relative to the light generating protein in the second expression cassette.

In yet a further embodiment, the invention is directed to a method of producing a transgenic, non-human mammal, such as a mouse. The mammal has at least one single-copy, non-essential gene in its genome. The method comprises

transfecting an embryonic stem cell of the mammal with a linear vector comprising

(a) a first selectable marker and a reporter expression cassette, the reporter expression cassette comprising a transcriptional promoter element operably linked to a light generating protein coding sequence, and

(b) targeting polynucleotide sequences homologous to a single-copy, non-essential gene in said mammal's genome, the targeting polynucleotide sequences

flanking (a), wherein (i) the length of the polynucleotide sequences are sufficient to facilitate homologous recombination between the vector and the single-copy, non-essential gene, and (ii) the transcriptional promoter element is heterologous to the single-copy, non-essential gene;

selecting embryonic stem cells which each have the first selectable marker and reporter expression cassette integrated into its genome;

injecting the embryonic stem cells into a host embryo,

implanting the embryo in a foster mother,

maintaining the foster mother under conditions which allow production of an offspring that is a transgenic, non-human mammal carrying the reporter expression cassette.

In certain embodiments, the offspring is capable of germline transmission of the reporter expression cassette and the method may further comprise breeding the offspring with a mammal which is substantially isogenic with the embryonic stem cells, such that the breeding yields transgenic F1 offspring carrying the reporter cassette. In particular embodiments, the method comprises breeding the first F1 offspring carrying the reporter cassette with a second F1 offspring carrying the reporter cassette, wherein the breeding yields transgenic F2 offspring carrying the reporter cassette.

In additional embodiments, the embryonic stems cells may be derived from a mouse having a dark coat color, the mammal substantially isogenic with the embryonic stem cells may have a light coat color, and/or the F2 offspring carrying the reporter cassette may have a light coat color. In particular embodiments, the embryonic stems cells are derived from a C57BL/6 mouse having a dark coat color, and the mammal substantially isogenic with the embryonic stem cells is a C57BL/6-Tyr C2j/+ mouse having a light coat color.

In still a further embodiment, the subject invention is directed to a vector for use in generating a transgenic non-human mammal, for example, a rodent such as a mouse. The mammal has at least one single-copy, non-essential gene in its genome. The vector comprises

(a) a first selectable marker and a reporter expression cassette, the reporter expression cassette comprising a transcriptional promoter element operably linked to a light generating protein coding sequence, and

(b) targeting polynucleotide sequences homologous to a single-copy, non-essential gene in the mammal's genome, the targeting polynucleotide sequences flanking (a), wherein (i) the length of the targeting polynucleotide sequences are sufficient to facilitate homologous recombination between the vector and the single-copy, non-essential gene, and (ii) the transcriptional promoter element is heterologous to the single-copy, non-essential gene.

In certain embodiments, the first selectable marker provides a positive selection and may be selected from the group consisting of neomycin phosphotransferase II, xanthine-guanine phosphoribosyltransferase, hygromycin-B-phosphotransferase, chloramphenicol acetyltransferase, and adeninephosphoribosyl transferase. Additionally, the transcriptional promoter element may be an inducible promoter, a repressible promoter, or a constitutive promoter, and may be selected from the group consisting of VEGF, VEGFR, and TIE2.

In alternative embodiments, the vector further comprises a second selectable marker and at least one target polynucleotide sequence is located between the second selectable marker and the first selectable marker. Additionally, the second selectable marker may provide a negative selection and may be selected from the group consisting of adenosine deaminase, thymidine kinase, and dihydrofolate reductase.

The vectors described above may be circular and may contain at least one restriction site whose cleavage results in a linear vector having the following arrangement of elements: target polynucleotide sequence - (a) - targeting polynucleotide sequences or target polynucleotide sequence - (a) - targeting polynucleotide sequences – (second selectable marker).

The coding sequences of the reporter expression cassette present in the vector may comprise codons that are optimal for expression in a host system into which the expression cassette is to be introduced. Additionally, the targeting polynucleotide sequences from single-copy, non-essential genes may be selected from the group consisting of vitronectin, *fosB*, and galactin 3.

The light-generating protein in the mammals and methods described above may be derived from either procaryotic or eucaryotic sources and, in particularly preferred embodiments, the light generating protein is a luciferase.

These and other embodiments of the present invention will be apparent to those of skill in the art in view of the teachings herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic depicting construction of the pTK53 vector. Polynucleotides encoding PGK-P, Neo and TK and 5' and 3' linkers are introduced into a pKS backbone to produce the vector designated pTK53.

Figure 2 is schematic depicting construction of the pTK-LucR and pTK-LucYG vectors. For pTK-LucR, a polynucleotide encoding LucR is introduced into pTK53. Thus, the pTK-LucR construct contains the PGK-P gene, a neomycin (Neo^r) gene, a thymidine kinase (TK) gene and sequence encoding red luciferase (Luc-R). For pTK-LucYG, a polynucleotide encoding LucYG is introduced into pTK53. Thus, the pTK-LucYG construct contains the PGK-P gene, a neomycin (Neo^r) gene, a thymidine kinase (TK) gene and a sequence encoding yellow-green luciferase (Luc-YG).

Figures 3A is a schematic depicting the vector pTKLR-Vn. Sequences homologous to the vitronectin gene are inserted into pTK-LucR such that they flank the Neo^r gene and the Luc-R coding sequence. Figure 3B is a schematic depicting targeting of the linearized pTKLR-Vn vector to the vintronectin chromosomal locus. The VEGF promoter is cloned into the polylinkers between Neo and Luc-R. Upon homologous recombination, the Neo-VEGF-LucR transgene is inserted into the Vn gene. In the figure, (A) shows the targeting vector pTKLR-Vn and (B) shows the mouse vitronectin gene. In the figure, Neo – neomycin resistance encoding sequences; TK – thymidine kinase encoding sequences; LucR – red luciferase from pGL3Red (Dr. Christopher Contag, Stanford University, Stanford, CA). Regions bearing Vn gene translational start and stop codons are indicated with arrows. Poly(A) sequences are placed upstream of the polylinker to prevent or minimize read-through translation. Figure 3C shows the nucleotide sequence of vitronectin (SEQ ID NO:38).

Figure 4A is a schematic depicting the vector pTKLG-Fos. Sequences homologous to the FosB gene are inserted into pTK-LucYG such that they flank the Neo^r gene and the Luc-YG coding sequence. Figure 4B shows the nucleotide sequence of FosB (SEQ ID NO:39).

Figure 5A is a schematic depicting targeting of the linearized pTKLG-Fos vector to the FosB chromosomal locus. The VEGFR2 promoter is cloned into the polylinkers between Neo and Luc-YG. Upon homologous recombination, the Neo-VEGFR2-LucYG

transgene will be inserted into a sequence associated with production of FosB. In the figure, (A) shows the targeting vector, and (B) shows the mouse target gene. In the figure, Neo – neomycin resistance encoding sequences; TK – thymidine kinase encoding sequences; LucYG – yellow green luciferase from pGL3-control vector (Promega, Madison, WI). Regions bearing FosB gene translational start and stop codons are indicated with arrows. Poly(A) sequences are placed upstream of the polylinker to prevent or minimize read-through translation. Figure 5B is a schematic depicting targeting of the linearized pTKLG-Fos vector to the FosB chromosomal locus. The TIE2 promoter is cloned into the polylinkers between Neo and Luc-R. Upon homologous recombination, the Neo-Tie2-LucYG transgene is inserted into the FosB gene. In the figure, (A) shows the targeting vector, and (B) shows the mouse target gene. In the figure, Neo – neomycin resistance encoding sequences; TK – thymidine kinase encoding sequences; LucYG – yellow green luciferase from pGL3-control vector (Promega). Regions bearing FosB gene translational start and stop codons are indicated with arrows. Poly(A) sequences are placed upstream of the polylinker to prevent or minimize read-through translation.

Figure 6 depicts PCR conditions for genomic screening for promoters useful in exemplary targeting constructs of the present invention.

Figure 7 depicts generation of targeted transgenic mice using the targeting vectors described herein.

Figure 8 depicts of schematic representation of Southern blot analysis of homologous DNA recombination between pTKLG-Fos targetting vector and the FosB gene.

Figure 9 depicts generation of targeted transgenic mice, using the targeting vectors described herein, and crosses using such transgenics as well as their offspring (F1, first generation; F2, second generation).

Figure 10 depicts crosses using transgenic mice of the present invention to generate dual luciferase transgenic mice.

MODES FOR CARRYING OUT THE INVENTION

Throughout this application, various publications, patents, and published patent applications are referred to by an identifying citation. The disclosures of these publications, patents, and published patent specifications referenced in this application are included to more fully describe the state of the art to which this invention pertains.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. *See, e.g.*, Sambrook, Fritsch, and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, (F.M. Ausubel et al. eds., 1987); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); PCR 2: A PRACTICAL APPROACH (M.J. McPherson, B.D. Hames and G.R. Taylor eds., 1995) and ANIMAL CELL CULTURE (R.I. Freshney. Ed., 1987).

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise. Thus, for example, reference to "an antigen" includes a mixture of two or more such agents.

Definitions

As used herein, certain terms will have specific meanings.

The terms "nucleic acid molecule" and "polynucleotide" are used interchangeably to and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. Non-limiting examples of polynucleotides include a gene, a gene fragment, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

A polynucleotide is typically composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); and thymine (T) (uracil (U) for thymine (T) when the polynucleotide is RNA). Thus, the term polynucleotide sequence is the alphabetical representation of a polynucleotide molecule. This alphabetical

representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching.

A "coding sequence" or a sequence which "encodes" a selected polypeptide, is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide, for example, *in vivo* when placed under the control of appropriate regulatory sequences (or "control elements"). The boundaries of the coding sequence are typically determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from viral, procaryotic or eucaryotic mRNA, genomic DNA sequences from viral or procaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence may be located 3' to the coding sequence. Other "control elements" may also be associated with a coding sequence. A DNA sequence encoding a polypeptide can be optimized for expression in a selected cell by using the codons preferred by the selected cell to represent the DNA copy of the desired polypeptide coding sequence. "Encoded by" refers to a nucleic acid sequence which codes for a polypeptide sequence, wherein the polypeptide sequence or a portion thereof contains an amino acid sequence of at least 3 to 5 amino acids, more preferably at least 8 to 10 amino acids, and even more preferably at least 15 to 20 amino acids from a polypeptide encoded by the nucleic acid sequence. Also encompassed are polypeptide sequences which are immunologically identifiable with a polypeptide encoded by the sequence.

Typical "control elements", include, but are not limited to, transcription promoters, transcription enhancer elements, transcription termination signals, polyadenylation sequences (located 3' to the translation stop codon), sequences for optimization of initiation of translation (located 5' to the coding sequence), translation enhancing sequences, and translation termination sequences. Transcription promoters can include inducible promoters (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), repressible promoters (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), and constitutive promoters.

"Expression enhancing sequences" typically refer to control elements that improve transcription or translation of a polynucleotide relative to the expression level in the absence of such control elements (for example, promoters, promoter enhancers, enhancer elements, and translational enhancers (e.g., Shine and Delagarno sequences)).

"Purified polynucleotide" refers to a polynucleotide of interest or fragment thereof which is essentially free, e.g., contains less than about 50%, preferably less than about 70%, and more preferably less than about 90%, of the protein with which the polynucleotide is naturally associated. Techniques for purifying polynucleotides of interest are well-known in the art and include, for example, disruption of the cell containing the polynucleotide with a chaotropic agent and separation of the polynucleotide(s) and proteins by ion-exchange chromatography, affinity chromatography and sedimentation according to density.

A "heterologous sequence" as used herein is typically refers to either (i) a nucleic acid sequence that is not normally found in the cell or organism of interest, or (ii) a nucleic acid sequence introduced at a genomic site wherein the nucleic acid sequence does not normally occur in nature at that site. For example, a DNA sequence encoding a polypetide can be obtained from yeast and introduced into a bacterial cell. In this case the yeast DNA sequence is "heterologous" to the native DNA of the bacterial cell. Alternatively, a promoter sequence from a Tie2 gene can be introduced into the genomic location of a *fosB* gene. In this case the Tie2 promoter sequence is "heterologous" to the native *fosB* genomic sequence.

A "polypeptide" is used in it broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or other peptidomimetics. The subunits may be linked by peptide bonds or by other bonds, for example ester, ether, etc. As used herein, the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. A peptide of three or more amino acids is commonly called an oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is typically called a polypeptide or a protein.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given promoter that is operably linked to a coding sequence (e.g., a reporter expression

cassette) is capable of effecting the expression of the coding sequence when the proper enzymes are present. The promoter or other control elements need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. For example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

"Recombinant" as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of the polynucleotide with which it is associated in nature; and/or (2) is linked to a polynucleotide other than that to which it is linked in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide. "Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such terms denoting procaryotic microorganisms or eucaryotic cell lines cultured as unicellular entities, are used interchangeably, and refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer DNA, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement to the original parent, due to accidental or deliberate mutation. Progeny of the parental cell which are sufficiently similar to the parent to be characterized by the relevant property, such as the presence of a nucleotide sequence encoding a desired peptide, are included in the progeny intended by this definition, and are covered by the above terms. An "isolated polynucleotide" molecule is a nucleic acid molecule separate and discrete from the whole organism with which the molecule is found in nature; or a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences (as defined below) in association therewith.

Techniques for determining nucleic acid and amino acid "sequence identity" also are known in the art. Typically, such techniques include determining the nucleotide sequence of the mRNA for a gene and/or determining the amino acid sequence encoded thereby, and comparing these sequences to a second nucleotide or amino acid sequence. In general, "identity" refers to an exact nucleotide-to-nucleotide or amino acid-to-amino

acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more sequences (polynucleotide or amino acid) can be compared by determining their "percent identity." The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M.O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, Nucl. Acids Res. 14(6):6745-6763 (1986). An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, WI) in the "BestFit" utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, WI). A preferred method of establishing percent identity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, CA). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence identity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + Swiss protein + Spupdate + PIR. Details of these programs can be found at the following internet address: http://www.ncbi.nlm.gov/cgi-bin/BLAST. When claiming sequences relative to sequences of the present invention, the desired degrees of sequence identity are at least

80%, 85-90%, preferably 92%, more preferably 95%, and even more preferably 98% sequence identity to the reference sequence (i.e., the sequences of the present invention).

Alternatively, the degree of sequence similarity between polynucleotides can be determined by hybridization of polynucleotides under conditions that form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 80%-85%, preferably at least about 85%-90%, more preferably at least about 90%-95%, and most preferably at least about 95%-98% sequence identity over a defined length of the molecules, as determined using the methods above. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning*, *supra*; *Nucleic Acid Hybridization*, *supra*.

Two nucleic acid fragments are considered to "selectively hybridize" as described herein. The degree of sequence identity between two nucleic acid molecules affects the efficiency and strength of hybridization events between such molecules. A partially identical nucleic acid sequence will at least partially inhibit a completely identical sequence from hybridizing to a target molecule. Inhibition of hybridization of the completely identical sequence can be assessed using hybridization assays that are well known in the art (e.g., Southern blot, Northern blot, solution hybridization, or the like, see Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (1989) Cold Spring Harbor, N.Y.). Such assays can be conducted using varying degrees of selectivity, for example, using conditions varying from low to high stringency. If conditions of low stringency are employed, the absence of non-specific binding can be assessed using a secondary probe that lacks even a partial degree of sequence identity (for example, a probe having less than about 30% sequence identity with the target molecule), such that, in the absence of non-specific binding events, the secondary probe will not hybridize to the target.

When utilizing a hybridization-based detection system, a nucleic acid probe is chosen that is complementary to a target nucleic acid sequence, and then by selection of appropriate conditions the probe and the target sequence "selectively hybridize," or bind, to each other to form a hybrid molecule. A nucleic acid molecule that is capable of hybridizing selectively to a target sequence under "moderately stringent" typically hybridizes under conditions that allow detection of a target nucleic acid sequence of at least about 10-14 nucleotides in length having at least approximately 70% sequence identity with the sequence of the selected nucleic acid probe. Stringent hybridization conditions typically allow detection of target nucleic acid sequences of at least about 10-14 nucleotides in length having a sequence identity of greater than about 90-95% with the sequence of the selected nucleic acid probe. Hybridization conditions useful for probe/target hybridization where the probe and target have a specific degree of sequence identity, can be determined as is known in the art (see, for example, Nucleic Acid Hybridization: A Practical Approach, editors B.D. Hames and S.J. Higgins, (1985) Oxford; Washington, DC; IRL Press).

With respect to stringency conditions for hybridization, it is well known in the art that numerous equivalent conditions can be employed to establish a particular stringency by varying, for example, the following factors: the length and nature of probe and target sequences, base composition of the various sequences, concentrations of salts and other hybridization solution components, the presence or absence of blocking agents in the hybridization solutions (e.g., formamide, dextran sulfate, and polyethylene glycol), hybridization reaction temperature and time parameters, as well as, varying wash conditions. The selection of a particular set of hybridization conditions is selected following standard methods in the art (see, for example, Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, (1989) Cold Spring Harbor, N.Y.).

A "vector" is capable of transferring gene sequences to target cells. Typically, "vector construct," "expression vector," and "gene transfer vector," mean any nucleic acid construct capable of directing the expression of a gene of interest and which can transfer gene sequences to target cells. Thus, the term includes cloning, and expression vehicles, as well as integrating vectors.

"Nucleic acid expression vector" or "expression cassette" refers to an assembly which is capable of directing the expression of a sequence or gene of interest. The

nucleic acid expression vector includes a promoter which is operably linked to the sequences or gene(s) of interest. Other control elements may be present as well. Expression cassettes described herein may be contained within a plasmid construct. In addition to the components of the expression cassette, the plasmid construct may also include a bacterial origin of replication, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA *(e.g.,* a M13 origin of replication), a multiple cloning site, and a "mammalian" origin of replication (e.g., a SV40 or adenovirus origin of replication).

An "expression cassette" comprises any nucleic acid construct capable of directing the expression of a gene/coding sequence of interest. Such cassettes can be constructed into a "vector," "vector construct," "expression vector," or "gene transfer vector," in order to transfer the expression cassette into target cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

"Luciferase," unless stated otherwise, includes prokaryotic and eukaryotic luciferases, as well as variants possessing varied or altered optical properties, such as luciferases that produce different colors of light (e.g., Kajiyama, N., and Nakano, E., *Protein Engineering* 4(6):691-693 (1991)).

"Light-generating" is defined as capable of generating light through a chemical reaction or through the absorption of radiation.

A "light generating protein" or "light-emitting protein" is a protein capable of generating light in the visible spectrum (between approximately 350 nm and 800 nm). Examples include bioluminescent protiens such as luciferases, *e.g.,* bacterial and firefly luciferases, as well as fluorescent proteins such as green fluorescent protein (GFP).

"Light" is defined herein, unless stated otherwise, as electromagnetic radiation having a wavelength of between about 300 nm and about 1100 nm.

"Animal" as used herein typically refers to a non-human mammal, including, without limitation, farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered.

A "transgenic animal" refers to a genetically engineered animal or offspring of genetically engineered animals. A transgenic animal usually contains material from at least one unrelated organism, such as from a virus, plant, or other animal. The "non-human animals" of the invention include vertebrates such as rodents, non-human primates, sheep, dogs, cows, amphibians, birds, fish, insects, reptiles, etc. The term "chimeric animal" is used to refer to animals in which the heterologous gene is found, or in which the heterologous gene is expressed in some but not all cells of the animal.

"Analyte" as used herein refers to any compound or substance whose effects (*e.g.*, induction or repression of a specific promoter) can be evaluated using the test animals and methods of the present invention. Such analytes include, but are not limited to, chemical compounds, pharmaceutical compounds, polypeptides, peptides, polynucleotides, and polynucleotide analogs. Many organizations (*e.g.*, the National Institutes of Health, pharmaceutical and chemical corporations) have large libraries of chemical or biological compounds from natural or synthetic processes, or fermentation broths or extracts. Such compounds/analytes can be employed in the practice of the present invention.

As used herein, the term "positive selection marker" refers to a gene encoding a product that enables only the cells that carry the gene to survive and/or grow under certain conditions. For example, plant and animal cells that express the introduced neomycin resistance (Neo^r) gene are resistant to the compound G418. Cells that do not carry the Neo^r gene marker are killed by G418. Other positive selection markers will be known to those of skill in the art. Typically, positive selection markers encode products that can be readily asssayed. Thus, positive selection markers can be used to determine whether a particular DNA construct has been introduced into a cell, organ or tissue.

"Negative selection marker" refers to gene encoding a product which can be used to selectively kill and/or inhibit growth of cells under certain conditions. Non-limiting examples of negative selection inserts include a herpes simplex virus (HSV)-thymidine kinase (TK) gene. Cells containing an active HSV-TK gene are incapable of growing in the presence of gangcylovir or similar agents. Thus, depending on the substrate, some gene products can act as either positive or negative selection markers.

The term "homologous recombination" refers to the exchange of DNA fragments between two DNA molecules or chromatids at the site of essentially identical nucleotide

sequences. It is understood that substantially homologous sequences can accommodate insertions, deletions, and substitutions in the nucleotide sequence. Thus, linear sequences of nucleotides can be essentially identical even if some of the nucleotide residues do not precisely correspond or align (see, above).

A "knock-out" mutation refers to partial or complete loss of expression of at least a portion the target gene. Examples of knock-out mutations include, but are not limited to, gene-replacement by heterologous sequences, gene disruption by heterologous sequences, and deletion of essential elements of the gene (e.g., promoter region, portions of a coding sequence). A "knock-out" mutation is typically identified by the phenotype generated by the mutation.

A "single-copy gene" as used herein refers to a gene represented in an organism's genome only by a single copy at a particular chromosomal locus. Accordingly, a diploid organism has two copies of the gene and both copies occur at the same chromosomal location.

A "gene" as used in the context of the present invention is a sequence of nucleotides in a genetic nucleic acid (chromosome, plasmid, etc.) with which a genetic function is associated. A gene is a hereditary unit, for example of an organism, comprising a polynucleotide sequence (e.g., a DNA sequence for mammals) that occupies a specific physical location (a "gene locus" or "genetic locus") within the genome of an organism. A gene can encode an expressed product, such as a polypeptide or a polynucleotide (e.g., tRNA). Alternatively, a gene may define a genomic location for a particular event/function, such as the binding of proteins and/or nucleic acids (e.g., phage attachment sites), wherein the gene does not encode an expressed product. Typically, a gene includes coding sequences, such as, polypeptide encoding sequences, and non-coding sequences, such as, promoter sequences, poly-adenlyation sequences, transcriptional regulatory sequences (e.g., enhancer sequences). Many eucaryotic genes have "exons" (coding sequences) interrupted by "introns" (non-coding sequences). In certain cases, a gene may share sequences with another gene(s) (e.g., overlapping genes).

"Isogenic" means two or more organisms or cells that are considered to be genetically identical. "Substantially isogenic" means two or more organisms or cells wherein, at the majority of genetic loci (e.g., greater than 99.000%, preferably more than 99.900%, more preferably greater than 99.990%, even more preferably greater than

99.999%), there exists genetic identity between the organisms or cells being compared. In the context of the present invention, two organisms (for example, mice) are considered to be "substantially isogenic" if, for example, inserted transgenes are the primary differences between the genetic make-up of the mice being compared. Further, if, for example, the genetic backgrounds of the mice being compared are the same with the exception that one of the mice has one or several defined mutation(s) (for example, affecting coat color), then these mice are considered to be substantially isogenic. An example of two strains of substantially isogenic mice are C57BL/6 and C57BL/6-Tyr C2j/+.

A "pseudogene" as used herein, refers to a type of gene sequence found in the genomes, typically, of eucaryotes, where the sequence closely resembles a known functional gene, but differs in that the pseudogene is non-functional. For example, the pseudogene sequence may contain several stop codons in what would correspond to an open reading frame in the functional gene. Pseudogenes can also have deletions or insertions relative to their corresponding functional gene. If, for example, in a genome there is a functional gene and a related pseudogene, the functional gene is considered to be a single-copy gene (accordingly, the pseudogene is considered to be single-copy as well).

A "non-essential gene" refers to a gene whose deletion, disruption, elimination, reduction of gene function, or mutation is non-lethal, and does not obviously adversely affect the organisms' ability to mature and reproduce. A "non-essential gene with no phenotype" refers to a non-essential gene whose deletion, disruption, elimination, reduction of gene function or mutation has no deleterious effect on the organism. Typically there are no phenotypically reflected gene dosage effects associated with modification of a non-essential gene with no phenotype -- for example, deletion, disruption or mutation of both copies of a non-essential gene with no phenotype in a diploid organism has essentially the same effect as deletion, disruption, or mutation of one of the two copies present in the diploid organism. In the context of the present invention, a non-essential gene is typically one whose function has been eliminated (e.g., by a deletion mutation) and such elimination of function was non-lethal and the organism developed, matured, and was able to reproduce.

The "native sequence" or "wild-type sequence" of a gene is the polynucleotide sequence that comprises the genetic locus corresponding to the gene, e.g., all regulatory and open-reading frame coding sequences required for expression of a completely functional gene product as they are present in the wild-type genome of an organism. The native sequence of a gene can include, for example, transcriptional promoter sequences, translation enhancing sequences, introns, exons, and poly-A processing signal sites. It is noted that in the general population, wild-type genes may include multiple prevalent versions that contain alterations in sequence relative to each other and yet do not cause a discernible pathological effect. These variations are designated "polymorphisms" or "allelic variations."

By "replacement sequence" is meant a polynucleotide sequence that is substituted for at least a portion of the native or wild-type sequence of a gene.

"Linear vector" or "linearized vector," as used herein, is a vector having two ends. For example, circular vectors, such as plasmids, can be linearized by digestion with a restriction endonuclease that cuts at a single site in the plasmid. Preferably, the targeting vectors described herein are linearized such that the ends are not within the targeting sequences.

General Overview

The present invention relates to vector constructs and methods of creating transgenic animals to be used, for example, as test systems. Methods of using the animals of the present invention include, but are not limited to, using these animals for studies involving tumor growth and other disease conditions. In the practice of the present invention, transgenic, non-human mammals are constructed where a single-copy, non-essential gene is replaced by a reporter expression cassette, preferably a gene encoding a light-generating protein, such as a luciferase-encoding gene, operably linked to a promoter. A variety of promoters are useful in the practice of the present invention, for example, promoters derived from genes associated with tumorigenesis or angiogenesis. Thus, an exemplary promoter can be one that is associated with proteins induced during tumorigenesis, for instance in the presence of tumor generating compounds or of tumors themselves. In this way, expression of the reporter cassette is induced in the animal when, for example, tumors are present, and progression of the

tumor can be evaluated by non-invasive imaging methods using the whole animal. Another exemplary promoter is one that is derived from a gene associated with angiogenesis. Because the promoter is linked to a reporter such as luciferase, non-invasive monitoring of the progression of angiogenesis is possible.

Various forms of the different embodiments of the invention, described herein, may be combined.

Non-invasive imaging and/or detecting of light-emitting conjugates in mammalian subjects was described in U.S. Patent No. 5,650,135, by Contag, et al., issued 22 July 1997. This imaging technology can be used in the practice of the present invention in view of the teachings of the present specification. In the imaging method, the conjugates contain a biocompatible entity and a light-generating moiety. Biocompatible entities include, but are not limited to, small molecules such as cyclic organic molecules; macromolecules such as proteins; microorganisms such as viruses, bacteria, yeast and fungi; eukaryotic cells; all types of pathogens and pathogenic substances; and particles such as beads and liposomes. In another aspect, biocompatible entities may be all or some of the cells that constitute the mammalian subject being imaged, for example, cells carrying the vector constructs of the present invention expressing a reporter expression cassette.

Light-emitting capability is conferred on the biocompatible entities by the conjugation of a light-generating moiety. Such moieties include fluorescent molecules, fluorescent proteins, enzymatic reactions giving off photons and luminescent substances, such as bioluminescent proteins. In the context of the present invention, light emitting capability is typically confered on target cells by having at least one copy of a light-generating protein, *e.g.*, a luciferase, present. In preferred embodiments, luciferase is operably linked to appropriate control elements which can facilitate expression of a polypeptide having luciferase activity. Substrates of luciferase can be endogenous to the cell or applied to the cell or system (e.g., injection into a transgenic mouse, having cells carrying a luciferase construct, of a suitable substrate for the luciferase, for example, luciferin). The conjugation may involve a chemical coupling step, genetic engineering of a fusion protein, or the transformation of a cell, microorganism or animal to express a light-generating protein.

Targeting Constructs

The targeting cassettes described herein typically include the following components: (1) a suitable vector backbone; (2) a polynucleotide encoding a light generating protein (3) a promoter operably linked to the light generating protein-encoding gene, wherein the promoter is heterologous to the light generating protein coding sequences; (4) a sequence encoding a positive selection marker; (5) insertion sites flanking the sequence encoding the positive selection marker and the polynucleotide encoding a light generating protein gene, for insertion of sequences which target a single-copy, non-essential chromosomal gene; and, optionally, (6) a sequence encoding a negative selection marker. Exemplary targeting constructs are shown in Figures 3B, 5A and 5B and described in Examples 1-3.

Suitable vector backbones generally include an F1 origin of replication; a colE1 plasmid-derived origin of replication; polyadenylation sequence(s); sequences encoding antibiotic resistance (*e.g.,* ampicillin resistance) and other regulatory or control elements. Non-limiting examples of appropriate backbones include: pBluescriptSK (Stratagene, La Jolla, CA); pBluescriptKS (Stratagene, La Jolla, CA) and other commercially available vectors.

In one aspect of the invention the light generating protein is luciferase. Luciferase coding sequences useful in the practice of the present invention include, but are not limited to, sequences obtained from *lux* genes (procaryotic genes encoding a luciferase activity) and *luc* genes (eucaryotic genes encoding a luciferase activity). A variety of luciferase encoding genes have been identified including, but not limited to, the following: B.A. Sherf and K.V. Wood, U.S. Patent No. 5,670,356, issued 23 September 1997; Kazami, J., et al., U.S. Patent No. 5,604,123, issued 18 February 1997; S. Zenno, et al, U.S. Patent No. 5,618,722; K.V. Wood, U.S. Patent No. 5,650,289, issued 22 July 1997; K.V. Wood, U.S. Patent No. 5,641,641, issued 24 June 1997; N. Kajiyama and E. Nakano, U.S. Patent No. 5,229,285, issued 20 July 1993; M.J. Cormier and W.W. Lorenz, U.S. Patent No. 5,292,658, issued 8 March 1994; M.J. Cormier and W.W. Lorenz, U.S. Patent No. 5,418,155, issued 23 May 1995; de Wet, J.R., et al, *Molec. Cell. Biol.* 7:725-737, 1987; Tatsumi, H.N., et al, *Biochim. Biophys. Acta* 1131:161-165, 1992; and Wood, K.V., et al, *Science* 244:700-702, 1989. Eukaryotic luciferase catalyzes a reaction using luciferin as a luminescent substrate to produce light,

whereas prokaryotic luciferase catalyzes a reaction using an aldehyde as a luminescent substrate to produce light.

Wild-type firefly luciferases typically have an emission maxima at about 550 nm. Numerous variants with differing emission maxima have also been studied. For example, Kajiyama and Nakano (*Protein Eng.* 4(6):691-693, 1991; U.S. Patent No. 5,330,906, issued 19 July 1994) teach five variant firefly luciferases generated by single amino acid changes to the *Luciola cruciata* luciferase coding sequence. The variants have emission peaks of 558 nm, 595 nm, 607 nm, 609 nm and 612 nm. A yellow-green luciferase with an emission peak of about 540 nm is commerically available from Promega, Madison, WI under the name pGL3. A red luciferase with an emission peak of about 610 nm is described, for example, in Contag et al. (1998) *Nat. Med.* 4:245-247 and Kajiyama et al. (1991) *Prot. Eng.* 4:691-693.

Positive selection markers include any gene which a product that can be readily asssayed. Examples include, but are not limited to, a hprt gene (Littlefield, J. W., Science 145:709-710 (1964)), a xanthine-guanine phosphoribosyltransferase (gpt) gene, or an adenosine phosphoribosyltransferase (aprt) gene (Sambrook et al., *supra*), a thymidine kinase gene (i.e "TK") and especially the TK gene of herpes simplex virus (Giphart-Gassler, M. et al., Mutat. Res. 214:223-232 (1989)), a nptII gene (Thomas, K. R. et al., Cell 51:503-512 (1987); Mansour, S. L. et al., Nature 336:348-352 (1988)), or other genes which confer resistance to amino acid or nucleoside analogues, or antibiotics, etc, for example, gene sequences which encode enzymes such as dihydrofolate reductase (DHFR) enzyme, adenosine deaminase (ADA), asparagine synthetase (AS), hygromycin B phosphotransferase, or a CAD enzyme (carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase). Addition of the appropriate substrate of the positive selection marker can be used to determine if the product of the positive selection marker is expressed, for example cells which do not express the positive selection marker nptII, are killed when exposed to the substrate G418 (Gibco BRL Life Technology, Gaithersburg, MD).

The targeting vector typically contains insertion sites for inserting targeting sequences (*e.g.*, sequences that are substantially homologous to the target sequences in the host genome where integration of the targeting vector/expression cassette is desired). These insertion sites are preferably included such that there are two sites, one site on

either side of the sequences encoding the positive selection marker, light generating protein (e.g., luciferase) and the promoter. Insertion sites are, for example, restriction endonuclease recognition sites, and can, for example, represent unique restriction sites. In this way, the vector can be digested with the appropriate enzymes and the targeting sequences ligated into the vector.

Optionally, the targeting construct can contain a polynucleotide encoding a negative selection marker. Suitable negative selection markers include, but are not limited to, HSV-tk (see, e.g., Majzoub et al. (1996) *New Engl. J. Med.* 334:904-907 and U.S. Patent No. 5,464,764), as well as genes encoding various toxins including the diphtheria toxin, the tetanus toxin, the cholera toxin and the pertussis toxin. A further negative selection marker gene is the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene for negative selection in 6-thioguanine.

Exemplary promoters and single-copy, non-essential genes for use in the vector constructs and methods of the present invention are described below.

Promoters

The targeting constructs and transgenic animals described herein contain a sequence encoding a light generating protein, e.g., luciferase, gene operably linked to a promoter. The promoter may be from the same species as the transgenic animal (*e.g.*, mouse promoter used in construct to make transgenic mouse) or from a different species (*e.g.*, human promoter used in construct to make transgenic mouse). The promoter can be derived from any gene of interest. In one embodiment of the present invention, the promoter is derived from a gene whose expression is induced during angiogenesis, for example pathogenic angiogenesis like tumor development. Thus, when a tumor begins to develop in a transgenic animal carrying a vector construct of the present invention, the promoter is induced and the animal expresses luciferase, which can then be monitored *in vivo*.

Exemplary promoters for use in the present invention are selected such that they are functional in a cell type and/or animal into which they are being introduced. Exemplary promoters include, but are not limited to, promoters obtained from the following mouse genes: vascular endothelial growth factor (VEGF) (VEGF promoter described in U.S. Patent No. 5,916,763; Shima et al. (1996) *J. Bio. Chem.* 271:3877-

3883; sequence available on NCBI under accession number U41383); VEGFR2, also known as Flk-1, (VEGFR-2 promoter described, for example, in Rönicke et al. (1996) *Circ. Res.* 79:277-285; Patterson et al. (1995) *J. Bio. Chem.* 270:23111-23118; Kappel et al. (1999) *Blood* 93:4282-4292; sequence available as accession number X89777 of NCBI database); Tie2, also known as Tek (Tie2 promoter described, for example, in Fadel et al. (1998) *Biochem. J.* 338:335-343; Schlaeger et al. (1995) *Develop.* 121:1089-1098; Schlager et al. (1997) *PNAS USA* 94:3058-3063). VEGF is a specific mitogen for EC *in vitro* and a potent angiogenic factor *in vivo*. In a tumorigenesis study, it was shown that VEGF was critical for the initial subcutaneous growth of T-47D breast carcinoma cells transplanted into nude mice, whereas other angiogenic factors, such as, bFGF can compensate for the loss of VEGF after the tumors have reached a certain size (Yoshiji, H., et al.,1997 Cancer Research 57: 3924-28). VEGF is a major mediator of aberrant EC proliferation and vascular permeability in a variety of human pathologic situation, such as, tumor angiogenesis, diabetic retinopathy and rheumatoid arthritis (Benjamin LE, et al.,1997 PNAS 94: 8761-66; Soker, S., et al.,1998 Cell 92: 735-745). VEGF is synthesized by tumor cells *in vivo* and accumulates in nearby blood vessels. Because leaky tumor vessels initiate a cascade of events, which include plasma extravasation and which lead ultimately to angiogenesis and tumor stroma formation, VEGF plays a pivotal role in promoting tumor growth (Dvorak, H.F., et al., 1991 J Exp Med 174:1275-8). VEGF expression was upregulated by hypoxia (Shweiki, D., et al., 1992 Nature 359: 843-5). VEGF is also upregulated by overexpression of v-Src oncogene (Mukhopadhyay. D., et al.,1995 Cancer Res. 15: 6161-5), c-SRC (Mukhopadhyay, D., et al., 1995 Nature 375: 577-81), and mutant ras oncogene (Plate, K.H., et al., 1992 Nature 359: 845-8). The tumor suppressor p53 downregulates VEGF expression (Mukhopadhyay. D., et al.,1995 Cancer Res. 15: 6161-5).

A number of cytokines and growth factors, including PGF and TPA (Grugel, S., et al., 1995 J. Biological Chem. 270: 25915-9), EGF, TGF-b, IL-1, IL-6 induce VEGF mRNA expression in certain type of cells (Ferrara, N., et al., 1997 Endocr. Rev. 18: 4-25). Kaposi's sarcoma-associated herpesvirus (KSHV) encoded a G-protein-coupled receptor, a homolog of IL-8 receptor, can activate JNK/SAPK and p38MAPK and increase VEGF production, thus causing cell transformation and tumorigenicity (Bais, C., et al., Nature 1998 391:86-9). VEGF overexpression in skin of transgenic mice

induces angiogenesis, vascularhyperpermeability and accelerated tumor development (Larcher, F., et al., Oncogene 1998 17:303-11).

VEGF-B (cDNA sequences available on databases) is a mitogen for EC and may be involved in angiogenesis in muscle and heart (Olofsson, B., et al., 1996 Proc Natl Acad Sci U S A 93:2576-81). Shown *in vitro*, binding of VEGF-B to its receptor VEGFR-1 leads to increased expression and activity of urokinase type plasminogen activator and plasminogen activator inhibitor, suggesting a role for VEGF-B in the regulation of extracellular matrix degradation, cell adhesion, and migration (Olofsson, B., et al., 1998 Proc Natl Acad Sci U S A 95:11709-14).

VEGF-C (see, e.g., U.S. Patent No. 5,916,763 and Shima et al., *supra*) may regulate angiogenesis of lymphatic vasculature, as suggested by the pattern of VEGF-C expression in mouse embryos (Kukk, E., et al., 1996 Development 122: 3829-37). Although VEGF-C is also a ligand for VEGFR-2, the functional significance of this potential interaction is unknown. Overexpression of VEGF-C in the skin of transgenic mice resulted in lymphatic, but not vascular, endothelial proliferation and vessel enlargement, suggesting the major function of VEGF-C is through VEGFR-3 rather than VEGFR-2 (Jeltsch M, et al., 1997 Science 276:1423-5). Shown by the CAM assay, VEGF and VEGF-C are specific angiogenic and lymphangiogenic growth factors, respectively (Oh, S.J., et al., (1997) *Devel. Biol.* 188: 96-109). VEGF-C overexpression in the skin of transgenic mice resulted in lymphatic, but not vascular, endothelial proliferation and vessel enlargement (Jeltsch M, et al., 1997 Science 276:1423-5).

VEGF-D (cDNA sequences available on databases) is a mitogen for EC. Given that VEGF-D can also activate VEGFR-3. it is possible that VEGF-D could be involved in the regulation of growth and/or differentiation of lymphatic endothelium (Achen, M.G., et al., 1998 Proc Natl Acad Sci U S A 95: 548-53). VEGF-D is induced by transcription factor c-Fos in mouse (Orlandini, M., 1996 PNAS 93: 11675-80).

VEGFR-1 signaling pathway may regulate normal endothelial cell-cell or cell matrix interactions during vascular development, as suggested by the knockout study (Fong, G.H., et al., 1995 Nature 376: 65-69). Although VEGFR-1 has a higher affinity to VEGF than VEGFR-2, it does not transduce the mitogenic signals of VEGF in ECs (Soker, S., et al.,1998 Cell 92: 735-745). VEGFR-2 (see, e.g., Rönicke et al., Patterson et al., Kappel et al. (1999), *supra*) appears to be the major transducer of VEGF signals in

EC that result in chemotaxis, mitogenicity and gross morphological changes in target cells (Soker, S., et al.,1998 Cell 92: 735-745). VEGFR-3 has an essential role in the development of the embryonic cardiovascular system before the emergence of lymphatic vessels, as shown by the knockout study (Dumont, D.J., et al., 1998 Science 282: 946-949). Neuropillin-1 (see, e.g., Soker et al. (1998) *Cell* 92:735-745) is a receptor for VEGF165. It can enhance the binding of VEGF165 to VEGFR-2 and VEGF165 mediated chemotaxis (Soker, S., et al.,1998 Cell 92: 735-745). Neuropillin1 overexpression in transgenic mice resulted in embryonic lethality. The embryos possessed excess capillaries and blood vessels. Dilated vessels and hemorrhage were also observed (Kitsukawa, T., et al., 1995 Development 121: 4309-18).

Further promoters of interest include, but are not limited to, the following. Ang2 is expressed only at predominant vascular remodeling sites, such as ovary, placenta, uterus (Maisonpierre, P.C., et al., 1997 Science 277: 55-60). In glioblastoma angiogenesis, Ang2 is found to be expressed in endothelial cells of small blood vessel and capilaries while Ang1 is expressed in glioblastoma tumor cells (Stratmann, A., 1998 Am J Pathol 153: 1459-66). Ang2 is up-regulated in bovine microvascular endothelial by VEGF, bFGF, cyrokines, hypoxia (Mandriota, S.J., 1998 Circ Res 83: 852-9). Ang2 transgenic overexpression disrupts angiogenesis, and is embryonic lethal (Maisonpierre, P.C., et al., 1997 Science 277: 55-60). Ang1 is widely expressed, less aboundant in heart and liver (Maisonpierre, P.C., et al., 1997 Science 277: 55-60). Ang1 is expressed in mesenchymal cells and may up-regulate the expression of Tie2 in the endothelial cells (Suri,C., et al., 1996 Cell 87: 1171-1180). Ang1 overexpression in the skin of transgenic mice produces larger, more numerous, and more highly branched vessels (Suri, C., et al., Science 1998 282:468-71). Tie2 (see, e.g., Fadel et al.; Schlaeger et al. (1995), and Schlager et al. (1997), *supra*) is edothelial cell specific, up-regulated during wound healing, follicle maturation (Puri, M.C., et al., 1995 EMBO J 14: 5884-91) and pathologic angiogenesis (Kaipainen, A., 1994 Cancer Research 54: 6571-77), such as, glioblastoma (Stratmann, A., 1998 Am J Pathol 153: 1459-66). Tie2 is also expressed in non-proliferating adult endothelium and edothelial cell lines (Dumont, D.J., et al. (1994) *Genes & Develop.* 8:1897-1909). A Tie2 activating mutation causes vascular dysmorphorgenesis (Vikkula M, et al., 1996 Cell 87: 1181-1190). Tie2 mutant overexpression in transgenic mice is embryonic lethal (Dumont, D.J., et al., *supra*).

Other promoters useful in the practice of the present invention include, by way of example, promoters derived from the sequences encoding the following polypeptide products: PTEN (dual specificity phosphatase); BAI (brain-specific angiogenesis inhibitor); KAI1 (KANGAI 1); catenin beta-1 (cadherin-associated protein, beta); COX2 (PTGS2 cyclooxygenase 2, a.k.a. prostaglandin-endoperoxide synthase 2); MMP2 (72 kDa Type IV-A collagenase); MMP9 (92 kDa type IV-B collagenase); TIMP2 (tissue inhibitor of metalloproteinase 2); and TIMP3 (tissue inhibitor of metalloproteinase 3).

PTEN is a tumor suppressor gene and encodes a protein of 403 amino acids. (Li et al. (1997) *Science* 275:1943-1946; DiCristofano et al. (1998) *Nature Genet.* 19:348-355). Overexpression of PTEN has been shown to inhibit cell migration and it is postulated that this protein may function as a tumor suppressor by negatively regulating cell interactions with the extracellular matrix or by negatively regulating the PI3K/PKB/Akt signaling pathway. (Tamura et al. (1998) *Science* 280:1614-1617; Stambolic et al. (1998) *Cell* 95:29-29). Mutations in PTEN have been detected in cancer cell lines and in the germline of patients having Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome (diseases and syndromes which are characterized by hyperplastic/dysplastic changes in the prostate, skin and colon and which are associated with an increased risk of certain cancers, for example, breast cancer, prostate cancer and colon cancer). (Marsh et al. (1998) *Hum. Molec. Genet.* 7:507-515; Marsh et al. (1998) *J. Med. Genet.* 35:881-885; Nelen et al. (1997) *Hum. Molec. Genet.* 6:1383-1387).

BAI1 protein is predicted to be 1,584 amino acids in length and includes an extracellular domain, an intracellular domain and a 7-span transmembrane region similar to that of the secretin receptor. (Nishimori et al. (1997) *Oncogene* 15:245-2150). The extracellular region of BAI1 has a single Arg-Gly-Asp (RGD) motif recognized by integrins and also has five sequences corresponding to the thrombospondin type I (accession number 188060) repeats that can inhibit angiogenesis includes by basic fibroblast growth factor (bFGF, accession number 134920). Shiratsuchi et al. (1997) *Cytogenet. Cell Genet.* 79:103-108, cloned 2 other brain-specific angiogenesis inhibiting genes, designated BAI2 (accession number 602683) and BAI 3 (accession number 602684). Thus, it is postulated that members of this gene family may play a role in suppression of glioblastoma.

KAI1 encodes a 267 amino acid protein which is a member of the leukocyte surface glyoprotein family. The protein has 4 hydrophobobic transmembrane domains and 1 large extracellular hydrophilic domain with three potential N-glycosylation sites. (Dong et al. (1995) *Science* 268:884-886). Molecular analysis of KAI1 is described, for example, in Dong et al. (1997) *Genomics* 41:25-32. KAI1 is a tumor metastasis suppressor gene that is capable of inhibiting the metastatic process in experimental animals. Expression of KAI1 is downregulated during tumor progression of prostate, breast, lung, bladder and pancreatic cancers in humans, apparently at the transcriptional or postranscriptional level. Mashimo et al. (1998) *PNAS USA* 95:11307-11311, found that the tumor suppressor gene p53 can directly inactivate the KAI1 gene by interacting with the region 5' to the coding sequence, suggesting a direct relationship between p53 and KAI1.

Catenin beta-1 is an adherens junction (AJ) protein, which are critical for establishing and maintaining epithelial cell layers, for instance during embryogenesis, wound healing and tumor cell metastasis. Molecular analysis, including description of sequence homology to plakoglobin (accession number 173325), homology to the drosophila gene "armadillo" and interactions with Lef1/Tcf DNA binding proteins, is described, for example, in Nollet et al. (1996) *Genomics* 32:413-424; McCrea et al. (1991) *Science* 254:1359-1361 and Korinek et al. (1997) *Science* 275:1784-1787. In addition, studies by Korinek et al., *supra* and Morin et al.(1997) *Science* 275:1787-1790, have indicated that APC (accession number 175100) negatively regulates catenin beta and that regulation of this protein is critical to the tumor suppressive effect of APC. Abnormally high levels of beta-catenin have been detected in certain human melanoma cell lines. (Rubinfeld et al. (1997) *Science* 275:1790-1792. Koch et al. (1999) *Cancer Res.* 59:269-273 report that childhood hepatoblastomas frequently carry a mutated degradation targeting box of the beta-catenin gene. Transgenic mice which express catenin beta under the control of an epidermal promoter undergo de novo hair morphogenesis and eventually these animals develop two types of tumors -- epithelioid cysts and trichofolliculomas. Gat et al. (1998) *Cell* 95:605-614.

COX2 encodes a cyclooxygenase and is a key regulator of prostaglandin synthesis. (Hla et al. (1992) *PNAS USA* 89:7384-7388; Jones et al. (1993) *J. Biol. Chem.* 268:9049-9054). In particular, COX2 is generally considered to be a mediator of

inflammation and overexpression of COX2 in rat epithelial cells results in elevated levels of E-cadherin and Bcl2. (Tsujii & DuBois (1995) *Cell* 83:493-501). In co-cultures of endothelial cells and colon carcinoma cells, cells that overexpress COX2 produce prostaglandins, proangiogenic factors and stimulate both endothelial migration and tube formation. (Tsujii et al. (1998) *Cell* 93:705-716). Experiments conducted using APC knock-out mice have demonstrated that animals homozygous for a disrupted COX2 locus develop significantly more adenomatous polyps. (Oshima et al. (1996) *Cell* 87:803-809). COX-2 "knock out" mice develop severe nephropathy, are susceptible to peritonitis, exhibit reduced arachidonic acid-induced inflammation and exhibit reduced indomethacin-induced gastric ulceration. (Morham et al. (1995) *Cell* 83:473-482; Langenbach et al. (1995) *Cell* 83:483-492). Female mice that are deficient in cyclooxygenase 2 exhibit multiple reproductive failures. (Lim et al. (1997) *Cell* 91:197-208.

MMP2 is a metalloproteinase that specifically cleaves type IV collagen. A C-terminal fragment of MMP2, termed PEX, prevents normal biding to alpha-V/beta-3 and disrupts angiogenesis and tumor growth. (Brooks et al. (1998) *Cell* 92:391-400).

MMP9 is a collagenase secreted from normal skin fibroblasts. MMP9 null mice exhibit an abnormal pattern of skeletal growth plate vascularization and ossification. (Vu et al. (1998) *Cell* 93:411-422).

TIMP2 is a collagenase and appears to play a major role in modulating the activity of interstitial collagenase and a number of connective tissue metalloendoproteases. (Stetler-Stevenson et al. (1989) *J. Biol. Chem.* 264:17372-17378). Unlike TIMP1 and TIMP3, TIMP2 is not upregulated by TPA or TGF-beta. (Hammani et al. (1996) *J. Biol. Chem.* 271:25498-25505).

TIMP3 (Wilde et al. (1994) *DNA Cell Biol.* 13:711-718) is localized in the extracellular matrix in both its glycosylated and unglycosylated forms. Studies of mutant TIMP3 proteins have demonstrated that C-terminal trunctions do not bind to the extracellular matrix. (Langton et al. (1998) *J. Biol. Chem.* 273:16778-16781).

As one of skill in the art will appreciate in view of the teachings of the present specification, promoter sequences can be easily derived and isolated from known polypeptide sequences or from cDNA or genomic sequences, using method known in the art in view of the teachings herein. An exemplary method of isolating promoter

sequences using cDNA is via a GenomeWalker® kit, commercially available from Clontech (Palo Alto, CA), and described on page 27 of the 1997-1998 Clontech catalog.

Targeting Sequences: Non-Essential Genes

Central to the present invention is the fact that the targeting constructs contain "targeting" sequences (flanking, for example, the light generating protein-encoding sequence and promoter) derived from a single-copy, non-essential gene. These targeting sequences in the construct act via homologous recombination to replace at least a portion of the non-essential gene in the genome with the light-generating protein-encoding (*e.g.*, luciferase-encoding) sequence operably linked to a promoter.

Non-limiting examples of targeting sequences for use in generating transgenic mice include sequences obtained from or derived from vitronectin, Fos B and galactin 3. A search of Mouse Knockout & Mutation Database (Genome Systems, Inc., St. Louis, MO) can be used to identify genes that have been knocked-out in mice where the generated knockout mice displayed no obvious defects. The chromosomal locus for all these genes can be used to target promoter-(light generating protein, e.g., luciferase) transgenes similar to what is described in Example 2. Single-copy, non-essential mouse genes identified in this manner include, but are not limited to, the following: Moesin (Msn), Doi Y., et al., J Biol Chem 1999, 274:2315–2321; Plasminogen activator inhibitor, type II (Planh2) and Planh1, Dougherty K.M., Proc Natl Acad Sci USA 1999, 96:686–691; Protein tyrosine phosphatase, receptor type, B (Ptprb), Elchebly et al. (1999) Science 283:1544–1548; Presenilin 1 (Psen1), Guo Q, et al. (1999) Proc Natl Acad Sci USA, 96:4125–4130; Protein kinase, mitogen-activated 9 (Prkm9) / SAPK/Erk/kinase 2 (Serk2), Kuan CY et al. (1999) Neuron 4:667–676; CD152 antigen (Cd152) / CD86 antigen (Cd86) / CD80 antigen (Cd80), Mandelbrot DA, et al. (1999) J Exp Med, 189:435–440; Poly (ADP-ribose) polymerase (Adprp), Masutani M, et al. (1999), Proc Natl Acad Sci USA 96:2301–2304; Sodium channel, nonvoltage-gated 1 beta (Scnn1b), Pradervand S, et al. (1999) Proc Natl Acad Sci USA 96:1732–1737; Nuclear receptor coactivator 1 (Ncoa1), Qi C, et al. (1999) Proc Natl Acad Sci USA 96:1585–1590; Decay accelerating factor 1 (Daf1), Sun X, et al. (1999) Proc Natl Acad Sci USA 1999, 96:628–633; Necdin (Ndn), Tsai TF, et al. (1999) Nat Genet 22:15–16; Relaxin (Rln); Zhao L, et al. (1999) Endocrinology 140:445–453; Adenylyl cyclase 8

(Adcy8), Abdel-Majid RM, et al. (1998) Nat Genet 19:289–291; Leukemia inhibitory factor (Lif), Bugga L, et al. (1998) J Neurobiol 36:509–524; Lectin, galactose binding, soluble 3 (Lgals3) and Lgals1, Calnot C, et al. (1998) Dev Dyn 211:306–313; Urokinase plasminogen activator receptor (Plaur) Carmeliet P, et al. (1998) J Cell Biol 140:233–245; Nitric oxide synthase 1, neuronal (Nos1), Chao DS, et al. (1998) J Neurochem 71:784–789; Homeo box A7 (Hoxa7), Chen F, et al. (1998) Mech Dev 77:49–57; Myosin light chain, phosphorylatable, cardiac ventricles (Mylpc) Chen J, et al. (1998) J Biol Chem 273:1252–1256; Homeo box B7 (Hoxb7),Chen F, et al. (1998) Mech Dev 77:49–57; Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha and beta (Nfkbia and Nfkbib), Cheng JD, et al. (1998) J Exp Med 6:1055–1062; Enolase 1, alpha non-neuron (Eno1), Couldrey C, et al. (1998) Dev Dyn 212:284–292; Xeroderma pigmentosum, complimentation group A (Xpa), De Vries A, et al. (1998) Exp Eye Res 1998, 67:53–59; Von Willebrand factor homolog (Vwf), Denis C, et al. (1998) Proc Natl Acad Sci USA 95:9524–9529; Lysosomal acid lipase 1 (Lip1), Du H, et al. (1998) Hum Mol Genet 7:1347–1354; UNC-5 homolog (C. elegans) 3 (Unc5h3), Eisenman LM, et al. (1998) J Comp Neurol 394:106–117; Protein phosphatase 1, regulatory (inhibitor) subunit 1B (Ppp1r1b), Fienberg AA, et al. (1998) Science 281:838–842; Myelin-associated glycoprotein (Mag) Fujita N, et al. (1998) J Neurosci 18:1970–1978; Paraoxonase 1 (Pon1), Furlong CE, et al. (1998) Neurotoxicology 19:645–650; Brain derived neurotrophic factor (Bdnf), Garek RR, et al. (1998) Laryngoscope 108:671–678; Neurotrophin 3 (Ntf3), Garek RR, et al. (1998) Laryngoscope 168:671–678; Myoglobin (Mb); Garry DJ, et al. (1998) Nature 395:905–908; Opioid receptor, mu (Oprm), Gaveriaux-Ruff C, et al. (1998) Proc Natl Acad Sci USA 95:6326–6330; Neuropeptide Y (Npy), Hollopeter G, et al. (1998) Int J Obes Relat Metab Disord 22:506–512; Procollagen, type I, alpha 1 (Cola1) Hormuzdi SG, et al. (1998) Mol Cell Biol 18:3368–3375; Centromere autoantigen B (Cenpb), Hudson DF, et al. (1998) J Cell Biol 141:309–319; Oculocerebrorenal syndrome of Lowe (ocrl), Janne PA, et al. (1998) J Clin Invest 101:2042–2053; arachidonate 12-lipoxygenase (Alox12) Johnson EN, et al. (1998) Proc Natl Acad Sci USA 95:3100–3105; H19 fetal liver mRNA (H19), Jones BK, et al. (1998) Genes Dev 12:2200–2207; Hepatocyte nuclear factor 3 gamma (winged helix transcription factor) (Hnf3g), Kaestner KH, et al. (1998) Mol Cell Biol 18:4245–4251; Bone morphogenetic protein 2 (Bmp2) / Bone

morphogenetic protein 7 (Bmp7) and Bmp5, Katagiri T, et al. (1998) Dev Genet 22:340–348; Intercellular adhesion molecule (Icam1), Ley K, et al. (1998) Circ Res 83:287–294; Glutamyl aminopeptidase (Enpep), Lin Q, et al. J Immunol 1998, 160:4681–4687; Prion protein (Prnp), Lipp HP, et al. Behav Brain Res 1998, 95:47–54; RAB3A, member RAS oncogene family (Rab3a), Lonart G, et al. Neuron 1998, 21:1141–1150; Potassium voltage gated channel, shaker related subfamily, member 4 (Kcna4), London B, et al., J Physiol (Lond) 1998, 509:171–182; Apurinic/apyrimidinic endonuclease (Apex), Ludwig DL, et al. Mutat Res 1998, 409:17–29; T-cell receptor gamma, variable 5 (Tcrg-V5), Mallick-Wood CA, et al. Science 1998, 279:1729–1733; Nuclear, factor, erythroid derived 2, like 2 (Nfe2l2), Martin F, et al. Blood 1998, 91:3459–3466; Interleukin 13 (Il13), McKenzie GJ, et al. Curr Biol 1998, 8:339–340; Sorbitol dehydrogenase 1 (Sdh1), Ng T, et al. Diabetes 1998, 47:961–966; Guanine nucleotide binding protein, alpha 11 (Gna11), Offermanns S, et al. EMBO J 1998, 17:4304–4312; Estrogen receptor alpha (Estra), Ogawa S, et al. Endocrinology 1998, 139:5070–5081; Integrin beta 2 (Itgb2), Intracellular adhesion molecule (Icam1) and CD34 antigen, Oliveira-dos-Santos AJ, et al. Eur J Immunol 1998, 28:2882–2892; Angiotensin receptor 1b (Agtr1b), Oliverio MI, et al. Proc Natl Acad Sci USA 1998, 95:15496–15501; Complement factor B (factor B), Pekna M, et al. Scand J Immunol 1998, 47:375–380; Centromere autoantigen B (Cenpb), Perez-Castro AV, et al. Dev Biol 1998, 201:135–143; Procollagen, type V, alpha 2 (Col5a2) / Fibrillin 1 (Fbn1), Phelps RG, et al. Mol Med 1998, 4:356–360; Plasminigen activator inhibitor, type 1 (Planh1), Pinsky DJ, et al. J Clin Invest 1998, 102:919–928, Carmeliet P, et al. J Clin Invest 1993, 92:2756–2760; Placentae and embryos oncofetal gene (Pem), Pitman JL, et al. Dev Biol 1998, 202:196–214; Postmeiotic segregation increased 1 (S. cerevisiae) (Pms1), Prolla TA, et al. Nat Genet 1998, 18:276–278; Prion protein, structural locus (Prn-p), Prusiner SB, et al. Proc Natl Acad Sci USA 1998, 90:10608–10612, Lledo P-M, et al. Proc Natl Acad Sci U S A 1996, 93:2403–2407, Sailer A, et al. Cell 1994, 77:967–968, Weissmann C, et al. Philos Trans R Soc Lond [Biol] 1994, 343:431–433, Bueler H, et al. Cell 1993, 73:1337–1347, Weissmann C, et al. Intervirology 1993, 35:164–175; NAD (P)H:quinone oxidoreductase, Radjendirane V, et al. J Biol Chem 1998, 273:7382–7389; Alpha tropomyosin (Tpm1), Rethinasamy P, et al. Circ Res 1998, 82:116–123; Goosecoid and Goosecoid-like (Gscl), Wakamiya M, et al. Hum Mol Genet 1998, 7:1835–1840, Saint-Pore B, et al. Hum Mol Genet 1998,

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Some preferred single-copy, non-essential genes with no phenotypes of the present invention include, but are not limited to, the following: Moesin (Msn), Doi Y., et al., J Biol Chem 1999, 274:2315–2321; Plasminogen activator inhibitor, type II (Planh2) and Planh1, Dougherty K.M., Proc Natl Acad Sci USA 1999, 96:686–691; Nuclear receptor coactivator 1 (Ncoa1), Qi C, et al. (1999) Proc Natl Acad Sci USA 96:1585–1590; Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha and beta (Nfkbia and Nfkbib), Cheng JD, et al. (1998) J Exp Med 6:1055–1062; H19 fetal liver mRNA (H19), Jones BK, et al. (1998) Genes Dev 12:2200–2207; Prion protein (Prnp), Lipp HP, et al. Behav Brain Res 1998, 95:47–54; Centromere autoantigen B (Cenpb), Perez-Castro AV, et al. Dev Biol 1998, 201:135–143; Placentae and embryos oncofetal gene (Pem), Pitman JL, et al. Dev Biol 1998, 202:196–214; Externally regulated phosphatase (Ptpn16), Dorfman K, et al. Oncogene 1996, 13:925–931; Transformation related protein 53 (Trp53), Ohashi M, et al. Jpn J Cancer Res 1996, 87:696–701; H1-0 histone (H1fv), Sirotkin AM, et al. Proc Natl Acad Sci U S A 1995, 92:6434–6438; Creatine kinase, mitochondrial 1, ubiquitous (Ckmt1), Steeghs K, et al. Biochim Biophys Acta 1995, 1230:130–138; Neuroblastoma ras oncogene (Nras), Umanoff H, et al. Proc Natl Acad Sci USA 1995, 92:1709–1713; Vitronectin (Vtn), Zheng X, et al. Proc Natl Acad Sci U S A 1995, 92:12426–12430; Vimentin (Vim),

Colucci GE, et al. Cell 1994, 79:679–694; Cellular retinoic acid binding protein I (Crabp1), Gorry P, et al. Proc Natl Acad Sci USA 1994, 91:9032–9036; Retinoic acid receptor beta2 (RARbeta2), Mendelsohn C, et al. Dev Biol 1994, 166:246–258; Retinoic acid receptor, alpha (Rara), Li E, et al. Proc Natl Acad Sci USA 1993, 90:1590–1594, Lufkin T, et al. Proc Natl Acad Sci USA 1993, 90:7225–7229; Lectin, galactose binding, soluble 1 (Lgals1), Poirier F, Robertson EJ. Development 1993, 119:1229–1236; Myogenic differentiation 1 (Myod1), Rudnicki MA, et al. Cell 1992, 71:383–390; and Tenascin C (Tnc), Saga Y, et al. Genes Dev 1992, 6:1821–1831.

In view of the guidance of the present specification, one of ordinary skill in the art can select similar, suitable, single-copy, non-essential genes in mice and other cell types/organisms.

Assembly of Targeting Cassettes

The targeting cassettes described herein can be constructed utilizing methodologies known in the art of molecular biology (see, for example, Ausubel or Maniatis) in view of the teachings of the specification. As described above, the targeting constructs are assembled by inserting, into a suitable vector backbone, polynuclotides encoding a reporter, such as a light-generating protein, *e.g.*, a luciferase gene, operably linked to a promoter of interest; a sequence encoding a positive selection marker; and, optionally a sequence encoding a negative selection marker. In addition, the targeting cassette contains insertion sites such that sequences targeting a single-copy, non-essential gene can be readily inserted to flank the sequence encoding positive selection marker and luciferase-encoding sequence.

A preferred method of obtaining polynucleotides, suitable regulatory sequences (*e.g.*, promoters) is PCR. General procedures for PCR as taught in MacPherson et al., PCR: A PRACTICAL APPROACH, (IRL Press at Oxford University Press, (1991)). PCR conditions for each application reaction may be empirically determined. A number of parameters influence the success of a reaction. Among these parameters are annealing temperature and time, extension time, Mg2+ and ATP concentration, pH, and the relative concentration of primers, templates and deoxyribonucleotides. Exemplary primers are described below in the Examples. After amplification, the resulting fragments can be

detected by agarose gel electrophoresis followed by visualization with ethidium bromide staining and ultraviolet illumination.

In one embodiment, PCR can be used to amplify fragments from genomic libraries. Many genomic libraries are commercially available. Alternatively, libraries can be produced by any method known in the art. Preferably, the organism(s) from which the DNA is has no discernible disease or phenotypic effects. This isolated DNA may be obtained from any cell source or body fluid (*e.g.*, ES cells, liver, kidney, blood cells, buccal cells, cerviovaginal cells, epithelial cells from urine, fetal cells, or any cells present in tissue obtained by biopsy, urine, blood, cerebrospinal fluid (CSF), and tissue exudates at the site of infection or inflammation). DNA is extracted from the cells or body fluid using known methods of cell lysis and DNA purification. The purified DNA is then introduced into a suitable expression system, for example a lambda phage.

Another method for obtaining polynucleotides, for example, short, random nucleotide sequences, is by enzymatic digestion. As described below in the Examples, short DNA sequences generated by digestion of DNA from vectors carrying genes encoding luciferase (yellow green or red).

Polynucleotides are inserted into vector genomes using methods known in the art. For example, insert and vector DNA can be contacted, under suitable conditions, with a restriction enzyme to create complementary or blunt ends on each molecule that can pair with each other and be joined with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of a polynucleotide. These synthetic linkers can contain nucleic acid sequences that correspond to a particular restriction site in the vector DNA. Other means are known and, in view of the teachings herein, can be used.

The final constructs can be used immediately (*e.g.*, for introduction into ES cells), or stored frozen (*e.g.*, at -20°C) until use. Preferably, the constructs are linearized prior to use, for example by digestion with suitable restriction endonucleases.

Transgenic Animals

The targeting constructs containing the light generating protein coding sequences (e.g., luciferase genes) are introduced into a pluripotent cell (*e.g.*, ES cell, Robertson, E. J., In: Current Communications in Molecular Biology, Capecchi, M. R. (ed.), Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), pp. 39-44). Suitable ES cells may be derived or isolated from any species or from any strain of a particular species. Although not required, the pluripotent cells are typically derived from the same species as the intended reciepient. ES cells may be obtained from commercial sources, from International Depositories (*e.g.*, the ATCC) or, alternatively, may be obtained as described in Robertson, E. J., *supra.* Examples of clonally-derived ES cells lines include 129/SVJ ES cells, RW-4 and C57BL/6 ES cells (Genome Systems, Inc.).

ES cells are cultured under suitable conditions, for example, as described in Ausubel et al., section 9.16, *supra.* Preferably, ES cells are cultured on stomal cells (such as STO cells (especially SNC4 STO cells) and/or primary embryonic fibroblast cells) as described by E. J. Robertson, *supra,* pp 71-112. Culture media preferably includes leukocyte inhibitory factor ("lif") (Gough, N. M. et al., Reprod. Fertil. Dev. 1:281-288 (1989); Yamamori, Y. et al., Science 246:1412-1416 (1989), which appears to help keep the ES cells from differentiating in culture. Stomal cells transformed with the gene encoding lif can also be used.

The targeting constructs are introduced into the ES cells by any method which will permit the introduced molecule to undergo recombination at its regions of homology, for example, micro-injection, calcium phosphate transformation, or electroporation (Toneguzzo, F. et al., Nucleic Acids Res. 16:5515-5532 (1988); Quillet, A. et al., J. Immunol. 141:17-20 (1988); Machy, P. et al., Proc. Natl. Acad, Sci. (U.S.A.) 85:8027-8031 (1988)). The construct to be inserted into the ES cell must first be in the linear form. Thus, if the knockout construct has been inserted into a vector as described above, linearization is accomplished by digesting the DNA with a suitable restriction endonuclease selected to cut only within the vector sequence and not within the knockout construct sequence. If the ES cells are to be electroporated to insert the construct, the ES cells and construct DNA are exposed to an electric pulse using an electroporation machine and following the manufacturer's guidelines for use. After electroporation, the ES cells are typically allowed to recover under suitable incubation conditions. The cells

are then cultured under conventional conditions, as are known in the art, and screened for the presence of the construct.

Screening and selection of those cells into which the targeting construct has been integrated can be achieved using the positive selection marker and/or the negative selection marker in the construct. In preferred embodiments, the construct contains both positive and negative selection markers. In one aspect, methods which rely on expression of the selection marker are used, for example, by adding the appropriate substrate to select only those cells which express the product of the positive selection marker or to eliminate those cells expressing the negative selection marker. For example, where the positive selection marker encodes neomycin resistance, G418 is added to the transformed ES cell culture media at increasing dosages. Similarly, where the negative selection marker is used, a suitable substrate (*e.g.*, gancyclovir if the negative selection marker encodes HSV-TK) is added to the cell culture. Either before or after selection using the appropriate substrate, the presence of the positive and/or negative selection markers in a recipient cell can also be determined by others methods, for example, hybridization, detection of radiolabelled nucleotides, PCR and the like. In preferred embodiments, cells having integrated targeting constructs are first selected by adding the appropriate substrate for the positive and/or negative selection markers. Cells that survive the selection process are then screened by other methods, such as PCR or Southern blotting, for the presence of integrated sequences.

After suitable ES cells containing the construct in the proper location have been identified, the cells can be inserted into an embryo, preferably a blastocyst. The blastocyts are obtained by perfusing the uterus of pregnant females. In one embodiment, the blastocyts are obtained from, for example, the FVB/N strain of mice and the ES cells are obtained from, for example, the C57BL/6 strain of mice. Suitable methods for accomplishing this are known to the skilled artisan, and are set forth by, e.g., Bradley et al., (1992) Biotechnology, 10:534-539. Insertion into the embryo may be accomplished in a variety of ways known to the skilled artisan, however a preferred method is by microinjection. For microinjection, about 10-30 ES cells are collected into a micropipet and injected into embryos that are at the proper stage of development to permit integration of the foreign ES cell containing the construct into the developing embryo.

The suitable stage of development for the embryo used for insertion of ES cells is species dependent, in mice it is about 3.5 days.

While any embryo of the right stage of development is suitable for use, it is preferred that blastocysts are used. In addition, preferred blastocysts are male and, furthermore, preferably have genes encoding a coat color that is different from that encoded by the genes ES cells. In this way, the offspring can be screened easily for the presence of the knockout construct by looking for mosaic coat color (indicating that the ES cell was incorporated into the developing embryo). Thus, for example, if the ES cell line carries the genes for black fur, the blastocyst selected will carry genes for white or brown fur.

After the ES cell has been introduced into the blastocyst, the blastocyst is typically implanted into the uterus of a pseudopregnant foster mother for gestation. Pseudopregnant females are prepared by mating with vasectomized males of the same species and successful implantation usually must occur within about 2-3 days of mating.

Offspring are screened initially for mosaic coat color where the coat color selection strategy has been employed. Southern blots and/or PCR may also be used to determine the presence of the sequences of interest. Mosaic (chimeric) offspring are then bred to each other to generate homozygous animals. Homozygotes and heterozygotes may be identified by Southern blotting of equivalent amounts of genomic DNA from mice that are the product of this cross, as well as mice that are known heterozygotes and wild type mice. Alternatively, Northern blots can be used to probe the mRNA to identify the presence or absence of transcripts encoding either the replaced gene, the light generating protein coding sequence (e.g., luciferase gene), or both. In addition, Western blots can be used to assess the level of expression of the luciferase protein with an antibody against the luciferase gene product. Finally, in situ analysis (such as fixing the cells and labeling with antibody) and/or FACS (fluorescence activated cell sorting) analysis of various cells from the offspring can be conducted using suitable (*e.g.*, anti-luciferase) antibodies to look for the presence or absence of the targeting construct.

In one embodiment of the present invention, the animals are from the C57BL/6 mouse strain. This strain develops a variety of tumors and has been used to develop a number of tumor cells lines, for example, B16 melanoma cells (including, B16F10, B16D5, and B16F1), Lewis lung carcinoma cells (including, LLC, LLC-h59), T241

mouse fibrosarcoma cells, RM-1 and pTC2 mouse prostate cancer cells, and MCA207 mouse sarcoma cells. These cell lines have been extensively used for *in vivo* tumor biology studies after injection into C57BL/6 mice. The generated targeted transgenic mice in the Examples are in C57BL/6 genetic background and these animals are suitable for injection or implantation of such tumor cells, as well as other tumor cells described in literature that are immunocompatent for C57BL/6 mice. Thus, the transgenic animals can then be used, for example, to monitor, *in vivo*, tumor progression (*e.g.*, growth) and the efficacy of therapies on tumor regression. For example, where the transgenic animal is tumor-susceptible, it is monitored for expression of a reporter, *e.g.*, luciferase, which is indicative of tumorigenesis and/or angiogenesis. The monitoring of expression of luciferase reporter expression cassettes using non-invasive whole animal imaging has been described (Contag, C. et al, U.S. Patent No. 5,650,135, July 22, 1997; Contag, P., et al, *Nature Medicine* 4(2):245-247, 1998; Contag, C., et al, *OSA TOPS on Biomedical Optical Spectroscopy and Diagnostics* 3:220-224, 1996; Contag, C.H., et al, *Photochemistry and Photobiology* 66(4):523-531, 1997; Contag, C.H., et al, *Molecular Microbiology* 18(4):593-603, 1995). Such imaging typically uses at least one photo detector device element, for example, a charge-coupled device (CCD) camera.

The transgenic animals described herein can also be used to determine the effect of an analyte (*e.g.*, therapy), for example on tumor progression where the promoter induces light generating protein (e.g., luciferase) expression when a tumor develops. Methods of administration of the analyte include, but are not limited to, injection (subcutaneously, epidermally, intradermally), intramucosal (such as nasal, rectal and vaginal), intraperitoneal, intravenous, oral or intramuscular. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. For example, the analyte of interest can be administered over a range of concentration to determine a dose/response curve. The analyte may be administered to a series of test animals or to a single test animal (given that response to the analyte can be cleared from the transgenic animal).

The following examples are intended only to illustrate the present invention and should in no way be construed as limiting the subject invention.

EXAMPLES

Example 1
Generating the Targeting Cassette and Vector

A. Creation of the Backbone Vector

pTK53: The 0.5 kb mouse phosphoglycerate kinase 1 promoter was amplified with PGK primers (PGKF, SEQ ID NO:1: ATCGAATTCTACCGGGTAGGGGAGGCGCTTT; PGKR, SEQ ID NO:2: GGCTGCAGGTCGAAAGGCCCGGAGATGAGG) using mouse genomic DNA (Genome Systems, Inc., St. Louis, MO) as template. This fragment was then double digested with EcoRI and PstI and cloned into the pKS vector (Stratagene, La Jolla, California) which was linearized with the same enzymes. The neomycin gene was amplified with NeoF (SEQ ID NO:3: ACCTGCAGCCAATATGGGATCGGCCATTGAAC) and NeoR (SEQ ID NO:4: GGATCCGCGGCCGCCCCCAGCTGGTTCTTTCCGCCTC) primers using pNTKV1907 (Stratagene) as a template. The 1.1 kb PCR fragment was double digested with PstI and BamHI and cloned into the pKS-PGK vector which was linearized with the same enzymes. This pKS-PGK-Neo vector was used to clone thymidine kinase gene as follows. Primers TKF (SEQ ID NO: 5: GGATCCTCTAGAGTCGAGCAGTGTGGTTTT) and TKR (SEQ ID NO:6: GAGCTCCCGTAGTCAGGTTTAGTTCGTCCG) were used to amplify the TK gene from pNTKV1907 (Stratagene). The amplified 2kb fragment was then digested with BamHI and SacI and cloned into pKS-PGK-Neo vector that was linearized with the same enzymes. This constructed vector was designated as pTK. A synthetic linker F5R5 was made after annealing of two primers (forward primer, F5R51, SEQ ID NO: 7: GTACATTTAAATCCTGCAGG; reverse primer, F5R52, SEQ ID NO:8: AGCTCCTGCAGGATTTAAAT). This linker was inserted between Asp718I and HindIII sites of pTK and the new construct was designated pTK5. A second synthetic linker F3R3 was made by annealing of two primers (forward primer, F3R31, SEQ ID NO:9: GGCCCGGGCTTAATTAATGCATCATATGGTACCGTTTAAACGCGGCCGCAAG CTTGTCGACGGCGCGCCGGCCGGCC; reverse primer, F3R32, SEQ ID NO:10:

GATCGGCCGGCCGGCGCGCCGTCGACAAGCTTGCGGCCGCGTTTAAACGGTA CCATATGATGCATTAATTAAGCCCG).). This linker was inserted between NotI and BamHI sites of pTK and the new construct was designated pTK53. Schematics of the vectors are shown are shown in Figure 1.

B. Introduction of Luciferase

pTK-LucYG and pTK-LucR: The yellow green luciferase gene was isolated from pGL3 vector (Promega) as a HindIII-SalI fragment and was cloned into pGK53 that was linearized with the same enzymes. The new construct was designated pTK-LucYG (8931 bp), shown in Figure 2.

The red luciferase gene was isolated from pGL3-red vector (Dr. Christopher Contag, Stanford University, Stanford, Calif.) as a HindIII-SalI fragment and was cloned into pGK53 that was linearized with the same enzymes. The new construct was designated pTK-LucR (8931 bp), shown in Figure 2.

Example 2

Insertion of Targeting Sequences

A. Generation of vitronectin targeting vector: The targeting construct pTKLR-Vn was generated by inserting vitronectin (VN) DNA sequences into pTK-LucR vector.

Vitronectin (VN) is an abundant glycoprotein present in plasma and the extracellular matrix of most tissues. In a previous study, it was shown that heterozygous mice carrying one normal and one null VN allele and homozygous null mice completely deficient in vitronectin demonstrate normal development, fertility, and survival. This suggests that VN is not essential for cell adhesion and migration during normal mouse development (Zheng, X., et al., Proc Natl Acad Sci U S A 1995 92:12426-30). Mouse vitronectin genomic DNA sequence of 5004 bp was obtained from GenBank database (Accession number X72091). Based on this sequence, a 1.63 kb 3'end vitronectin fragment was amplified (reverse primer, VN1R, SEQ ID NO:11: CTGTATTTAAATCTGCCCACCCTATTCAGGACAGTAGTC; forward primer, VN1F, SEQ ID NO:12: CCAATGCATCAACCCAGCCAGGAGGAGTGCG) using mouse C57BL/6 genomic DNA as template (Genome Systems, Inc., St. Louis, MO).

This fragment was digested with *SwaI* and *NsiI* and cloned into pTK-LucR (linearized with *SwaI* and *SbfI*). This construct was designated as pTK-LucR3. Subsequently, a 2.35 kb 5'end vitronectin fragment was amplified (reverse primer, VN2R, SEQ ID NO:13: AACGCGTCGACTTCGGAGATGTTTCGGGGATAACCAGG; forward primer, VN2F, SEQ ID NO:14: TTGGCGCGCCCCATAGAGAAGAGACACCAAAGGCACGCTC) using mouse C57BL/6 genomic DNA as template. This fragment was digested with *SalI* and *AscI* and cloned into pTK-LucR vector that was linearized with *SalI* and *AscI*. This construct was designated as pTKLR-Vn. Figure 3 shows the restriction map of pTKLR-Vn vector. The polylinker between the neomycin gene and red luciferase gene is used to insert the VEGF promoter or other promoters of interests. The predicted homologous recombination between pTKLR-Vn and vitronectin gene is illustrated in Figure 3A. Upon insertion of the VEGF-LucR transgene cassette, the endogenous vitronectin gene is destroyed. Figure 3B shows the genomic DNA sequence of VN.

B. Generation of Fos targeting vector: The targeting construct pTKLG-Fos was generated by inserting FosB DNA sequences into pTK-LucYG vector.

FosB is one of the members of the Fos family. It plays a functional role in transcriptional regulation. It has been shown that FosB mice are born at a normal frequency, are fertile and present no obvious phenotypic or histologic abnormalities (Gruda et al (1996) *Oncogene* 12:2177-2185). A 28.8 kb genomic region that contains mouse FosB DNA sequence was obtained from GenBank database (Accession number AF093624).

Using this sequence, a 1.71 kb 5'end FosB fragment was amplified (forward primer, FosB1F, SEQ ID NO:15: CTGTATTTAAATCCCGTTTCTCACTGTGCCTGTGTC; reverse primer, FosB1R, SEQ ID NO:16: GTCTCCTGCAGGCTTCCTCCTCCTTGTTCCTTGCG) using mouse C57BL/6 genomic DNA as template. This fragment was digested with *SwaI* and *SbfI* and cloned into pTK-LucYG vector that was linearized with *SwaI* and *SbfI*. This construct was designated as pTK-LucYG3. Subsequently, a 1.58 kb 3'end FosB fragment was amplified (forward primer, FosB2F, SEQ ID NO:17: AACGCGTCGACGGATGGGATTGACCCCCAGCCCTC; reverse primer, FosB2R,

SEQ ID NO:18: TTGGCGCGCCCCTTGCCTCCACCTCTCAAATGC) using mouse C57BL/6 genomic DNA as template. This fragment was digested with *SalI* and *AscI* and cloned into pTK-LucYG vector that was linearized with *SalI* and *AscI*. This construct was designated as pTKLG-Fos (Figure 4). The polylinker between the neomycin gene and red luciferase gene is used to insert the VEGFR2 promoter (Example 3, Figure 5A), Tie2 promoter (Example 3, Figure 5B), as well as, other promoters of interests. The predicted homologous recombination between the targeting vector bearing the VEGFR2 promoter (Figure 5A) or the Tie2 promoter (Figure 5B) and FosB gene is also illustrated. As shown in the Figures, the VEGFR2-LucYG transgene cassette and Tie2-LucYG transgene cassette is inserted downstream of FosB gene translational stop signal. Therefore, the targeted transgenic mice should still have a functional FosB gene while expressing the transgenes. Figure 4B showns the DNA sequence of FosB.

Example 3

Insertion of Promoter Sequences of Interest

A. pTKLR-Vn/VEGF: Mouse VEGF genomic DNA sequence of 2240 bp that contains a partial VEGF promoter region was obtained from GenBank (accession number: U41383). Accordingly, primers were designed to amplify a 0.69 kb (VF1-VR1A; Table 1) and a 0.98 kb fragment (VF2-VR2; Table 1). It was confirmed that each pair of primers can amplify the predicted product using mouse129SvJ genomic DNA as template.

Table 1

Name	Seq Id No	Sequence
VF1	19	ACCTC ACTCT CCTGT CTCCC CTGAT TCCCA A
VR1A	20	GCTCT GGCGG TCACC CCCAA AAGCA
VF2	21	CCCTT TCCAA GACCC GTGCC ATTTG AGC
VR2	22	ACTTT GCCCC TGTCC CTCTC TCTGT TCGC
KF1	23	GCTGC GTCCA GATTT GCTCT CAGAT GCG
KR1	24	TTCTC AGGCA CAGAC TCCTT CTCCG TCCCT
KF2	25	CAGAT GGACG AGAAA ACAGT AGAGG CGTTG GC
KR2	26	GAGGA CTCAG GGCAG AAAGA GAGCG
TF3	27	AGCTT AGCCT GCAAG GGTGG TCCTC ATCG
TF2	28	CAAAT GCACC CCAGA GAACA GCTTA GCCTG C
TR1	29	GCTTT CAACA ACTCA CAACT TTGCG ACTTC CCG

Conditions for PCR amplification are shown in Figure 6. These primers were used for PCR screening of mouse 129/SvJ genomic DNA BAC (bacterial artificial chromosome) library (Genome Systems, Inc., St. Louis, MO). The library, on average, contained inserts of 120 kb with sizes ranging between 50 kb to 240 kb. A large genomic DNA fragment that contained VEGF promoter region was obtained. Southern blot analysis was performed to map the VEGF promoter region. A unique HindIII restriction site was mapped approximately 7.8 kb upstream of the ATG translational start codon of the VEGF gene. The sequences between HindIII and ATG translational start codon are inserted into the polylinker of pTKLR-Vn vector to finish the construction of targeting vector that contains VEGF-LucR transgene (Figure 3A).

B. pTKLG-Fos/VEGFR2

A mouse VEGFR2 genomic DNA sequence of 1079 bp which contains a partial VEGFR2 promoter region was published previously (Ronicke, et al., (1996) *Cir. Res.* 79:277-285). Accordingly, primers were designed to amplify a 0.45 kb (KF1-KR1A; Table 1) and a 0.58 kb fragment (KF2-KR2; Table 1). It was confirmed that each pair of primers can amplify the predicted product using mouse129SvJ genomic DNA as

template. DNA sequences for these primers are shown in Table 1 above and PCR amplification conditions are shown in Figure 6. These primers were used for PCR screening of mouse 129/SvJ genomic DNA BAC library. From the screening, a BAC clone that contained a large genomic DNA fragment was obatined. Based on the published restriction map of VEGFR2, a 4.5 kb HindIII-XbaI fragment that covers the VEGFR2 promoter region was subcloned from the VEGFR2 BAC clone into the pBluescriptSK vector (Stratagene, La Jolla, CA) that was linearized with HindIII and XbaI. The VEGFR2 promoter sequences of 4.1 kb, spanning from a HindIII site to the ATG translational start codon, are subcloned into the polylinker of pTKLG-Fos vector to construct the targeting vector that contains VEGFR2-LucYG transgene (Figure 5A).

C. pTKLG-Fos/Tie2

Mouse Tie2 genomic DNA sequence of 477 bp which contain a partial Tie2 promoter region has been published previously (Fadel et al (1998) *Biochem. J.* 330(Pt. 1):335-343). Accordingly, primers were designed to amplify a 0.45 kb (TF3-TR1; Table 1) and a 0.47 kb fragment (TF2-TR1; Table 1). It was confirmed that each pair of primers amplified the predicted product using mouse129SvJ genomic DNA as template. DNA sequences for these primers are shown in Table 1 above. PCR amplification conditions are shown in Figure 6. These primers are used for PCR screening of mouse 129SvJ genomic DNA BAC library. From the screening, a BAC clone containing a large genomic DNA fragment of the Tie2 promoter region was obtained. Based on the published Tie2 genomic DNA restriction map (see, Dumont et al., *supra*), a 10.5 kb Asp718-EcoRV fragment containing the Tie2 promoter region was subcloned ifrom the Tie2 BAC clone into the pSK vector that was linearized with Asp718 and EcoRV. The Tie2 promoter sequences of about 6.8 kb, spanning from the Asp718 site to the ATG translational start codon is subcloned into the polylinker of pTKLG-Fos vector to construct the Tie2-LucYG targeting vector (Figure 5B).

Example 4

Generation of Transgenic Mice Carrying the Constructs of the Present Invention

A. General Procedure: Figure 7 depicts a generalized description of generation of transgenic mice using the targeted transgenic vectors described in Example 3. Details regarding embryonic stem (ES) cell culture, transfection, blastocyst injection and implantation to a pseudopregnant foster are described, for example, in Hogan et al (1994) "Manipulating the Mouse Embryo, A Laboratory Manual. Second Edition", Cold Spring Harbour Laboratory Press.

After construction the targeted transgenic construct are transfected into C57BL/6 embryonic stem (ES) cells. (Genome System Inc., Genome Systems, Inc., St. Louis, MO) through electroporation. The antibiotic G418 is used to select for cells in which the DNA construct containing the Neo gene is integrated, either randomly or by homologous recombination. The nucleoside analog gancyclovir is converted by TK to a cytotoxic derivative. DNA that has integrated by homologous recombination lose the TK gene and are resistant to the drug, whereas cells that have incorporated the DNA randomly are likely to retain the TK gene. Thus, cells containing random integrations into a chromosomal location that allows the expression of the TK gene are killed. The G418 and gancyclovir resistant clones are then be screened by PCR and Southern blot analysis and those that have homologous DNA recombination is used for FVB/N blastocyst injection (Genome System, Inc.). Between 4-16 blastocysts are transferred to the uterus of a pseudopregnant foster mother. The pups are typically born 17 days after the transfer. Either random bred mice or F1 hybrid mice make suitable recipients. Females of certain random-bred stocks (*e.g.*, CD1 mice, from Charles River Laboratories) have very large ampullae, which makes oviduct transfer easier. These mice also generally make good mothers. Alternatively, F1 hybrid females (*e.g.*, B6 x CBA F1) can be used as recipients. Although their ampullae are smaller, make exceptionally good mothers,rearing litters as small as two pups. See, for example, Hogan et al. (1994), *supra.*

B. Screening for homol g us DNA recombination p sitive ES cells

1). pTKLG-Fos/VEGFR2: Analysis of homologous DNA recombination between pTKLG-Fos/VEGFR2 targeting vector and the FosB gene is carried out using Southern blot analysis as shown in Figure 8. Genomic DNA prepared from G418 resistant ES cells is digested with PvuII and probed with probe A to confirm the 5'end DNA recombination. PvuII digestion of DNA bearing homologous recombination reveals two separate bands of 8.2 and 4.0 kb, whereas digestion of DNA from homologous recombination negative clones reveals only the 8.2 kb band. The 3'end of DNA recombination is tested by hybridizing NotI digested DNA with probe B. NotI digestion of DNA bearing homologous recombination will reveal two separate bands of >8.2kb and 5.0 kb, whereas digestion of DNA from homologous recombination negative clones will only reveal the >8.2 kb band. Once homologous DNA recombination is confirmed, positive clones is selected for FVB/N blastocyst injection.

2) pTKLG-Fos/Tie2: Analysis of homologous DNA recombination between pTKLG-Fos/Tie2 targeting vector and the FosB gene is analyzed by Southern blot in a similar manner as described above for pTKLG-Fos/VEGFR2. Once homologous DNA recombination is confirmed, positive clones are selected for FVB/N blastocyst injection.

3) PTKLR-Vn/VEGF: Analysis of homologous DNA recombination between pTKLR-Vn/VEGF targeting vector and the vitronectin gene is analyzed by PCR. DNA primers designed according to the predicted homologous recombination, are listed in Table 2.

Table 2
PCR primers for analysis of homologous DNA recombination between pTKLR-Vn/VEGF targeting vector and the vitronectin gene

5'end primers	
F51 5'- CCCAGTGTCTCTGATTTAGGGAGAGCACCTGAG -3'	SEQ ID NO:30
R51 5'- CCAGACTGCCTTGGGAAAAGCGCCTC -3'	SEQ ID NO:31
F52 5'- CAGTGAGAGTCTTCTCTGTCCCTCAATCGGTTCTG -3'	SEQ ID NO:32
R52 5'- TGGATGTGGAATGTGTGCGAGGCCAG -3'	SEQ ID NO:33
3'end primers	
F31 5'- AATCAAAGAGGCGAACTGTGTGTGAGAGGTCC -3'	SEQ ID NO:34
R31 5'- CGGCTCCCCAAAATGTGGAAGCAAGC -3'	SEQ ID NO:35
F32 5'- GAATCCATCTTGCTCCAACACCCCAACATC -3'	SEQ ID NO:36
R32 5'- CGCCTCCTCTCCCCAGTCTCCCCTTG -3'	SEQ ID NO:37

Primers F51-R51 and F52-R52 amplify a 1799 bp and a 1841 bp DNA fragment respectively from the 5'end of the transgene that is integrated into the vitronectin site through homologous DNA recombination, whereas primers F31-R31 and F32-R32 amplify a 3549 bp and a 3428 bp DNA fragment respectively from the 3'end of the transgene that is integrated into the vitronectin site through homologous DNA recombination. Clones that allow successful amplification of both the 5'end and 3'end of the integrated transgene are selected for FVB/N blastocyst injection.

C. Analysis of chimeric mice

The pups developed from injected blastocysts contain chimeras, as can be identified by their agouti coat color when an ES cell derived from a mouse having a dark coat color (e.g., C57BL/6) is injected into the blastocyst of a light coat color animal (e.g., FVB/N, genotype B/B). DNA analysis (*e.g.*, Southern blotting, PCR) is conducted to further confirm the presence of the transgene in these pups as described above in Section B. These animals may be obtained commerically, for example from The Jackson Laboratory, Bar Harbor, MN.

D. Generating targeted transgenic C57BL/6 mice with white coat color

Breeding of the chimeric mice generates homozygous targeted transgenic mice, as depicted in Figure 9. The targeted mice are used to monitor gene expression through the measurement of luciferase mediated light emission from the mice. In a preferred embodiment, the targeted mouse has a light coat color (e.g., white coat color), because the black colored coat (an example of a dark coat color) of C57BL/6 mice can absorb light emitted from the body and may interfere the sensitivity of the bioluminescence assay. An inbred mouse strain C57BL/6-Tyr C2j/+ strain (Jackson Laboratory, Bar Harbor, MN) is available for this purpose. This strain of mice have white color coat, yet they still have the same genetic background as C57BL/6 mice except that the gene responsible for the black coat color is mutated. Unfortunately, C57BL/6-Tyr C2j/+ ES cells are not currently available. Therefore, the designed breeding program illustrated in Figure 9 is aimed to generate mice that are homozygous for the target transgene and have white coat color. C57BL/6 ES cells are prepared as described above and introduced into a suitable blastocyst (*e.g.*, from the FVB/N strain of mice). The blastocysts are implanted into a foster mother. Chimeric mice are shown in Figure 9 as white animals with black and green patches. Chimeric animals are bred with C57BL/6-Tyr C2j/+ mice to create F1 hybrids. Subsequent breeding of the F1 hybrids generates several type of mice, including the one that is homozygous for the target transgene and has a white coat color (shown in Figure 9 as b/b; L/L), which is used for *in vivo* gene regulation monitoring.

A C57BL/6 mouse and a C57BL/6-Tyr C2j/+ mouse are considered to be substantially isogenic. Accordingly, the method of the present invention exemplified in Figure 9 provides a means for generating breeding groups of substantially isogenic mice in a selected genetic background carrying at least one transgene of interest.

E. Dual luciferase targeted transgenic mice

As described above, two targeting vectors are generated. PTKLR-Vn carries a red luciferase gene and is targeted into vitronectin locus. PTKLG-Fos carries a yellow-green luciferase gene and is targeted into FosB locus. A number of promoters, including VEGF promoter, VEGFR2 promoter, and Tie2 promoter are cloned into these vectors, as described above. Subsequently three type of targeted transgenic mice are generated.

VEGF mice carry VEGF promoter-red luciferase transgene (VEGF-LucR) integrated into vitronectin locus. VEGFR2 mice carry VEGFR2 promoter-yellow-green luciferase (VEGFR2-LucYG) transgene integrated into FosB locus. Tie2 mice carry Tie2 promoter-yellow-green luciferase (Tie2-LucYG) transgene integrated into FosB locus. Through a breeding program illustrated in Figure 10, dual luciferase targeted transgenic mice are produced, carrying both of the VEGF-LucR and the VEGFR2-LucYG transgenes. The degradation of luciferin by yellow-green luciferase and red luciferase generates lights that emit at 540 nM and 610 nM respectively. These wavelengths of light are measured individually using a photo-counting camera (intensified CCD). Therefore, both VEGF expression and VEGFR2 expression, for example, can then be monitored in the same mouse at the same time.

As is apparent to one of skill in the art, various modification and variations of the above embodiments can be made without departing from the spirit and scope of this invention. These modifications and variations are within the scope of this invention.

CLAIMS

What is claimed is:

1. A vector for use in generating a transgenic non-human mammal, said mammal having at least one single-copy, non-essential gene in its genome, said vector comprising

(a) a first selectable marker and a reporter expression cassette, said reporter expression cassette comprising a transcriptional promoter element operably linked to a light-generating protein coding sequence, and

(b) targeting polynucleotide sequences homologous to a single-copy, non-essential gene in said mammal's genome, said targeting polynucleotide sequences flanking (a), wherein (i) the length of the targeting polynucleotide sequences are sufficient to facilitate homologous recombination between the vector and the single-copy, non-essential gene, and (ii) said transcriptional promoter element is heterologous to the single-copy, non-essential gene.

2. The vector of claim 1, wherein said first selectable marker provides a positive selection.

3. The vector of claim 2, wherein said first selectable marker is selected from the group consisting of neomycin phosphotransferase II, xanthine-guanine phosphoribosyltransferase, hygromycin-B-phosphotransferase, chloramphenicol acetyltransferase, and adeninephosphoribosyl transferase.

4. The vector of claim 3, wherein the first selectable marker is neomycin phosphotransferase II.

5. The vector of any of claims 1-4, further comprising a second selectable marker, wherein at least one target polynucleotide sequence is located between said second selectable marker and (a).

6. The vector of claim 5, wherein said second selectable marker provides a negative selection.

7. The vector of claim 6, wherein said second selectable marker is selected from the group consisting of adenosine deaminase, thymidine kinase, and dihydrofolate reductase.

8. The vector of any of claims 1-7, wherein the transcriptional promoter element is selected from the group consisting of an inducible promoter, a repressible promoter, and a constitutive promoter.

9. The vector of claim 8, wherein the transcriptional promoter element is selected from the group consisting of VEGF, VEGFR, and TIE2.

10. The vector of any of claims 1-9, wherein the sequences encoding the light-generating protein are obtained from either procaryotic or eucaryotic sources.

11. The vector of claim 10, wherein the light generating protein is a luciferase.

12. The vector of any of claims 1-11, wherein said reporter expression cassette further comprises other control elements.

13. The vector of claim 12, wherein said control elements are selected from the group consisting of transcription enhancer elements, transcription termination signals, polyadenylation sequences, sequences for optimization of initiation of translation, and translation termination sequences.

14. The vector of any of claims 1-11, wherein said vector is circular.

15. The vector of claim 14, wherein said vector contains at least one restriction site whose cleavage results in a linear vector having the following arrangement of elements: targeting polynucleotide sequence - (a) - targeting polynucleotide sequences.

16. The vector of claim 5, wherein said vector is circular.

17. The vector of claim 16, wherein said vector contains at least one restriction site whose cleavage results in a linear vector having the following arrangement of elements: target polynucleotide sequence - (a) - targeting polynucleotide sequences – (second selectable marker).

18. The vector of any of claims 1-17, wherein the coding sequences of the reporter expression cassette comprise codons that are optimal for expression in a host system into which the expression cassette is to be introduced.

19. The vector of claim 18, wherein said mammal is a rodent.

20. The vector of claim 19, wherein said mammal is a mouse.

21. The vector of any of claims 1-20, wherein said targeting polynucleotide sequences from single-copy, non-essential genes are selected from the group consisting of vitronectin, *fosB*, and galactin 3.

22. A method of producing a transgenic, non-human mammal, said mammal having at least one single-copy, non-essential gene in its genome, comprising

transfecting an embryonic stem cell of said mammal with a linear vector comprising

(a) a first selectable marker and a reporter expression cassette, said reporter expression cassette comprising a transcriptional promoter element operably linked to a light-generating protein coding sequence, and

(b) targeting polynucleotide sequences homologous to a single-copy, non-essential gene in said mammal's genome, said targeting polynucleotide sequences flanking (a), wherein (i) the length of the polynucleotide sequences are sufficient to facilitate homologous recombination between the vector and the single-copy, non-essential gene, and (ii) said transcriptional promoter element is heterologous to the single-copy, non-essential gene;

selecting embryonic stem cells which each have said first selectable marker and reporter expression cassette integrated into its genome;

injecting said embryonic stem cells into a host embryo,

implanting said embryo in a foster mother,

maintaining said foster mother under conditions which allow production of an offspring that is a transgenic, non-human mammal carrying said reporter expression cassette.

23. The method of claim 22, wherein said offspring is capable of germline transmission of said reporter expression cassette.

24. The method of claim 23 further comprising breeding said offspring with a mammal, wherein the mammal is substantially isogenic with the embryonic stem cells, wherein said breeding yields transgenic F1 offspring carrying said reporter cassette.

25. The method of claim 24, further comprising breeding a first F1 offspring carrying said reporter cassette with a second F1 offspring carrying said reporter cassette, wherein said breeding yields transgenic F2 offspring carrying said reporter cassette.

26. The method of any of claims 22-25, wherein said mammal is a mouse.

27. The method of claim 26, wherein said embryonic stems cells are derived from a mouse having a dark coat color.

28. The method of claim 27, wherein said mammal substantially isogenic with the embryonic stem cells has a light coat color.

29. The method of claim 28, wherein said F2 offspring carrying said reporter cassette has a light coat color.

30. The method of claim 29, wherein said embryonic stems cells are derived from a C57BL/6 mouse having a dark coat color, and said mammal substantially

isogenic with the embryonic stem cells is a C57BL/6-Tyr C2j/+ mouse having a light coat color.

31. The method of any of claims 22-30, wherein the light generating protein is a luciferase.

32. A transgenic, non-human mammal, comprising at least one single-copy, non-essential gene in its genome, wherein (i) at least a portion of at least one single-copy, non-essential gene is replaced by polynucleotide sequences heterologous to the gene, such that the gene cannot produce a functional gene product, and (ii) said polynucleotide sequences comprise a first expression cassette which has been introduced into said mammal or an ancestor of said mammal, at an embryonic stage, said first expression cassette comprising

a first selectable marker,

a first transcriptional promoter element heterologous to the gene, and

light-generating protein coding sequences, wherein said light-generating protein coding sequences are operably linked to said promoter element.

33. The transgenic, non-human mammal of claim 32, wherein the single-copy, non-essential gene is selected from the group consisting of vitronectin, *fosB*, and galactin 3.

34. The transgenic, non-human mammal of any of claims 32-33, wherein the first selectable marker is selected from the group consisting of neomycin phosphotransferase II, xanthine-guanine phosphoribosyltransferase, hygromycin-B-phosphotransferase, chloramphenicol acetyltransferase, and adeninephosphoribosyl transferase.

35. The transgenic, non-human mammal of any of claims 32-34, wherein the first transcriptional promoter element is selected from the group consisting of an inducible promoter, a repressible promoter, and a constitutive promoter.

36. The transgenic, non-human mammal of claim 35, wherein the first transcriptional promoter element is selected from the group consisting of VEGF, VEGFR, and TIE2.

37. The transgenic, non-human mammal of any of claims 32-36, wherein said expression cassette further comprises other control elements.

38. The transgenic, non-human mammal of claim 37, wherein said control elements are selected from the group consisting of transcription enhancer elements, transcription termination signals, polyadenylation sequences, sequences for optimization of initiation of translation, and translation termination sequences.

39. The transgenic, non-human mammal of any of claims 32-38, wherein said non-human mammal comprises a second single-copy, non-essential gene in its genome, wherein (i) at least a portion of the second single-copy, non-essential gene is replaced by polynucleotide sequences heterologous to the second gene, such that the second gene cannot produce a functional gene product, and (ii) said polynucleotide sequences comprise a second expression cassette which has been introduced into said mammal or an ancestor of said mammal, at an embryonic stage, said first expression cassette comprising

a second selectable marker,

a second transcriptional promoter element heterologous to the second gene, and

light-generating protein coding sequences, wherein said light-generating protein coding sequences are operably linked to said second promoter element.

40. The transgenic, non-human mammal of claim 39, wherein the first transcriptional promoter element in the first expression cassette is different from the second transcriptional promoter element in the second expression cassette.

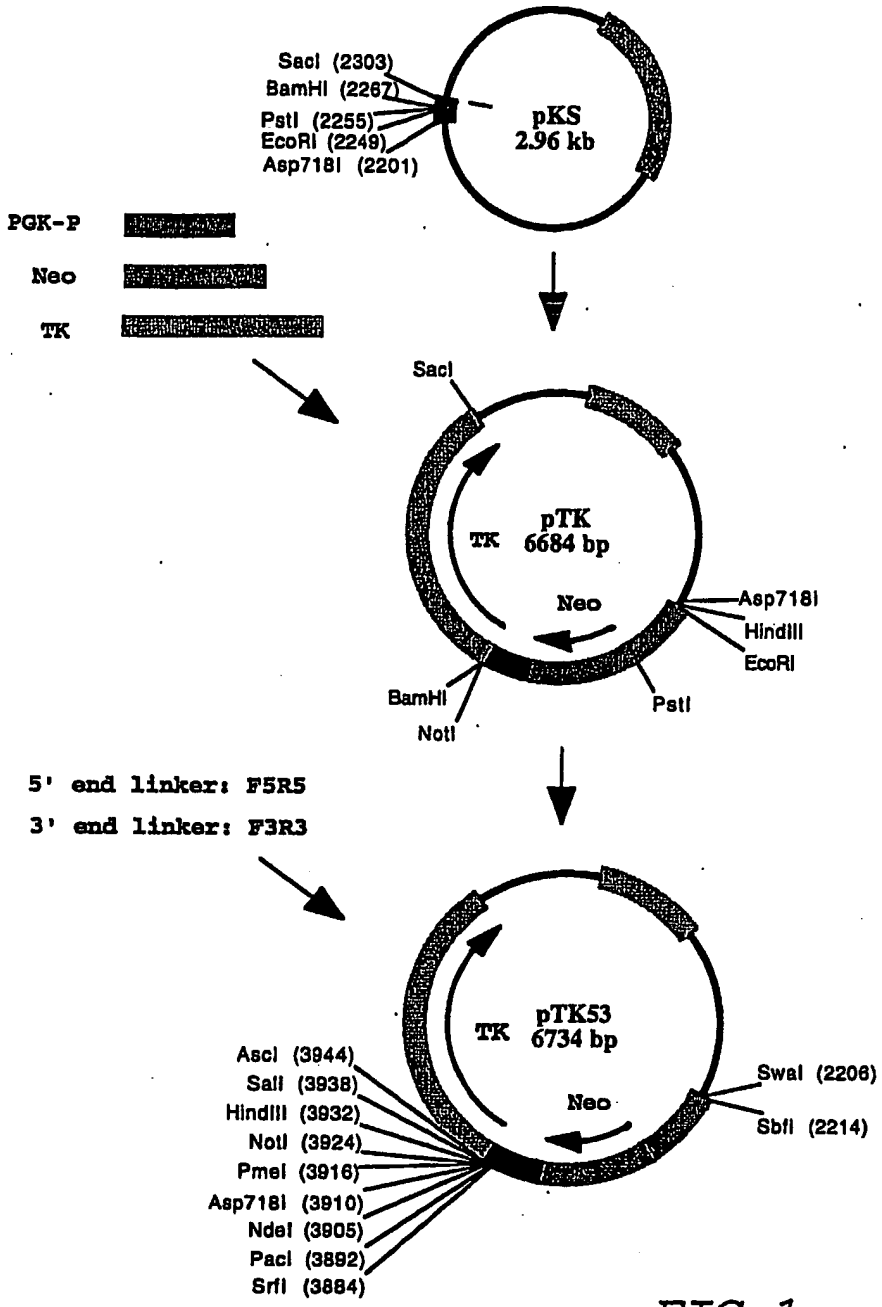
41. The transgenic, non-human mammal of claim 40, wherein the light-generating protein in the first expression cassette can produce a different color of light relative to the light-generating protein in the second expression cassette.

42. The transgenic, non-human mammal of any of claims 32-41, said mammal being a rodent.

43. The transgenic, non-human mammal of 42, said rodent being a mouse.

44. The transgenic, non-human mammal of any of claims 32-43, wherein the sequences encoding the light-generating protein are obtained from either procaryotic or eucaryotic sources.

45. The transgenic, non-human mammal of claim 44 wherein the light-generating protein is a luciferase.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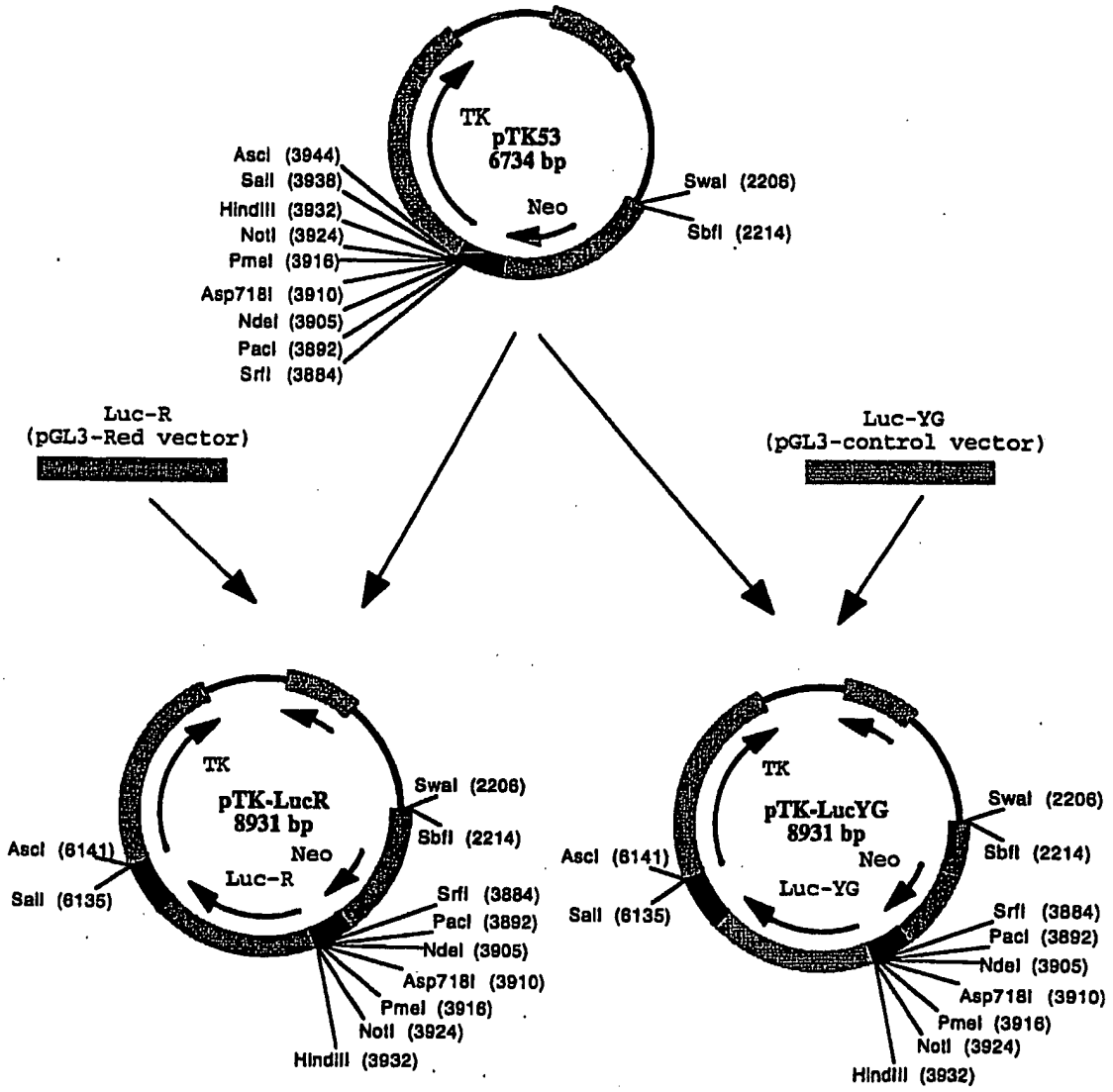
SwaI (2206)
SbfI (2214)

FIG. 1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PacI (3892)
SrfI (3884)
SwaI (2206)
SbfI (2214)
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(pGL3-Red vector)
Luc-YG
(pGL3-control vector)
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Luc-R
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SalI (6135)
SwaI (2206)
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PmeI (3916)
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FIG. 2

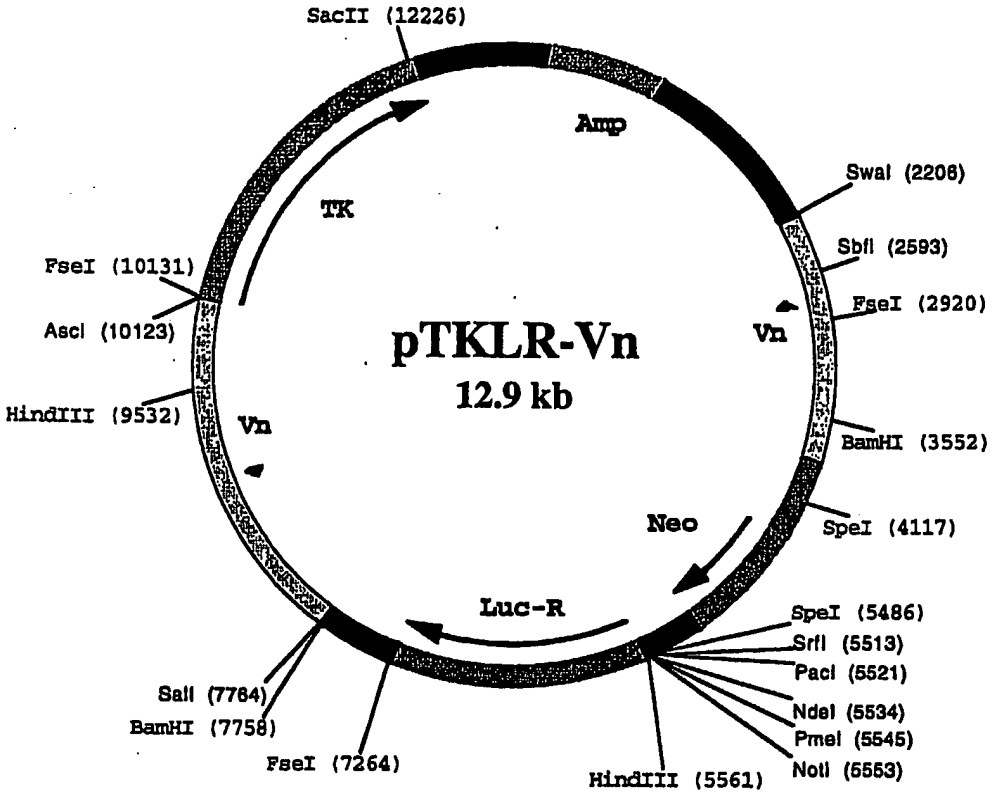
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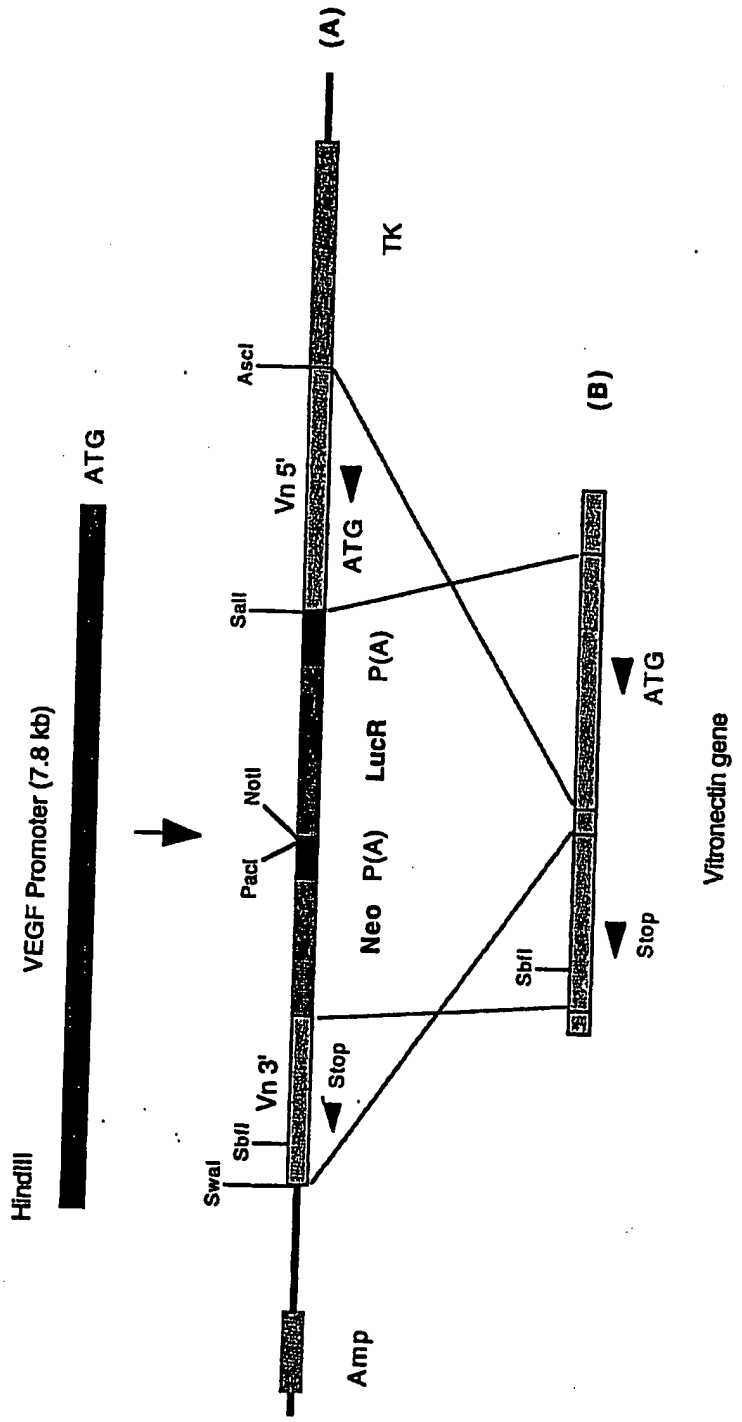
FIG. 3A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AscI
Vn 5'
ATG
SalI
P(A)
LucR
NotI
PacI
Neo
P(A)
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Stop
SbfI
SwaI
Amp
ATG
VEGF Promoter (7.8 kb)
HindIII
(B)
ATG
Stop
SbfI
Vitronectin gene

FIG.3B

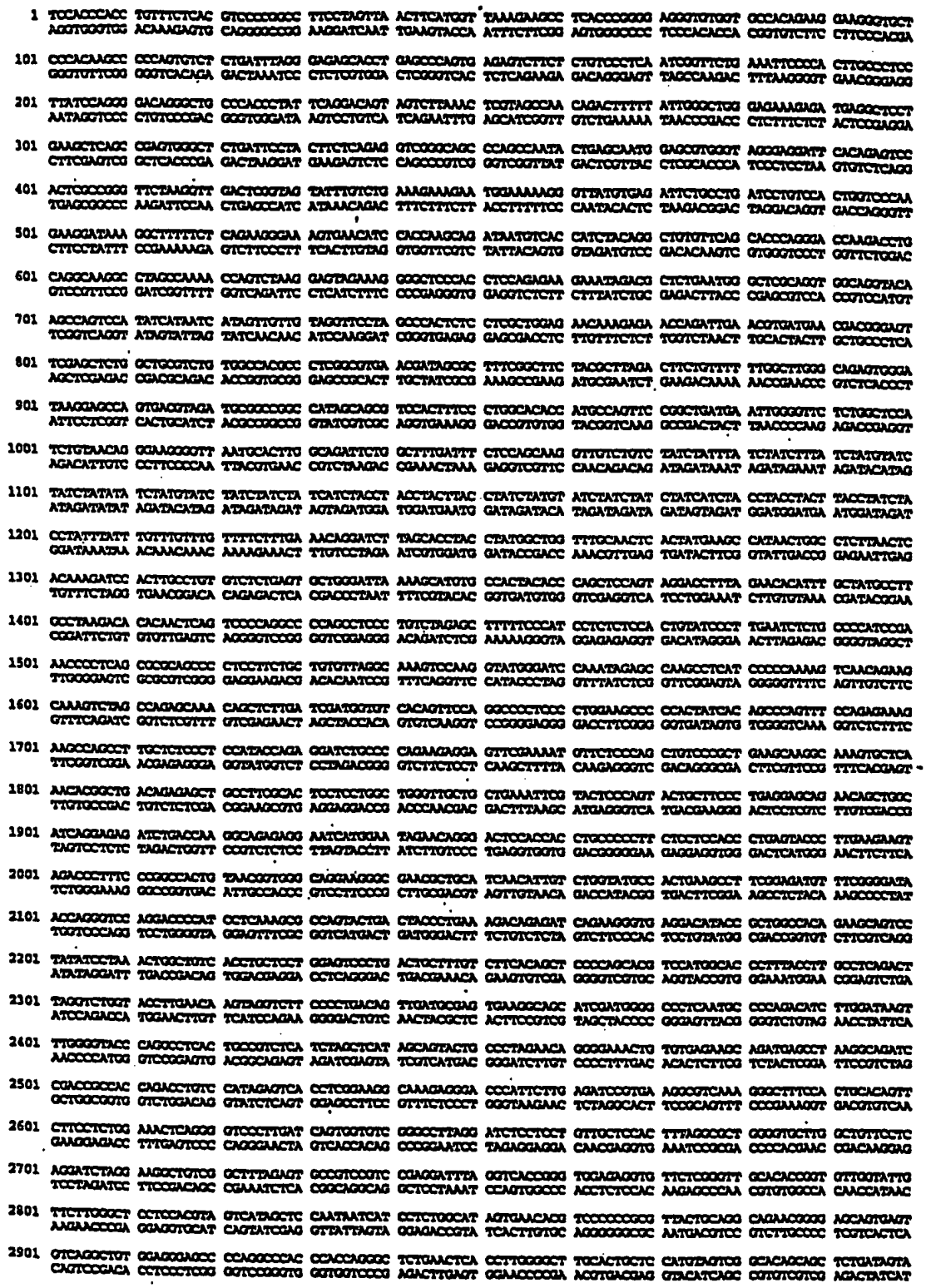


FIG. 3C-1

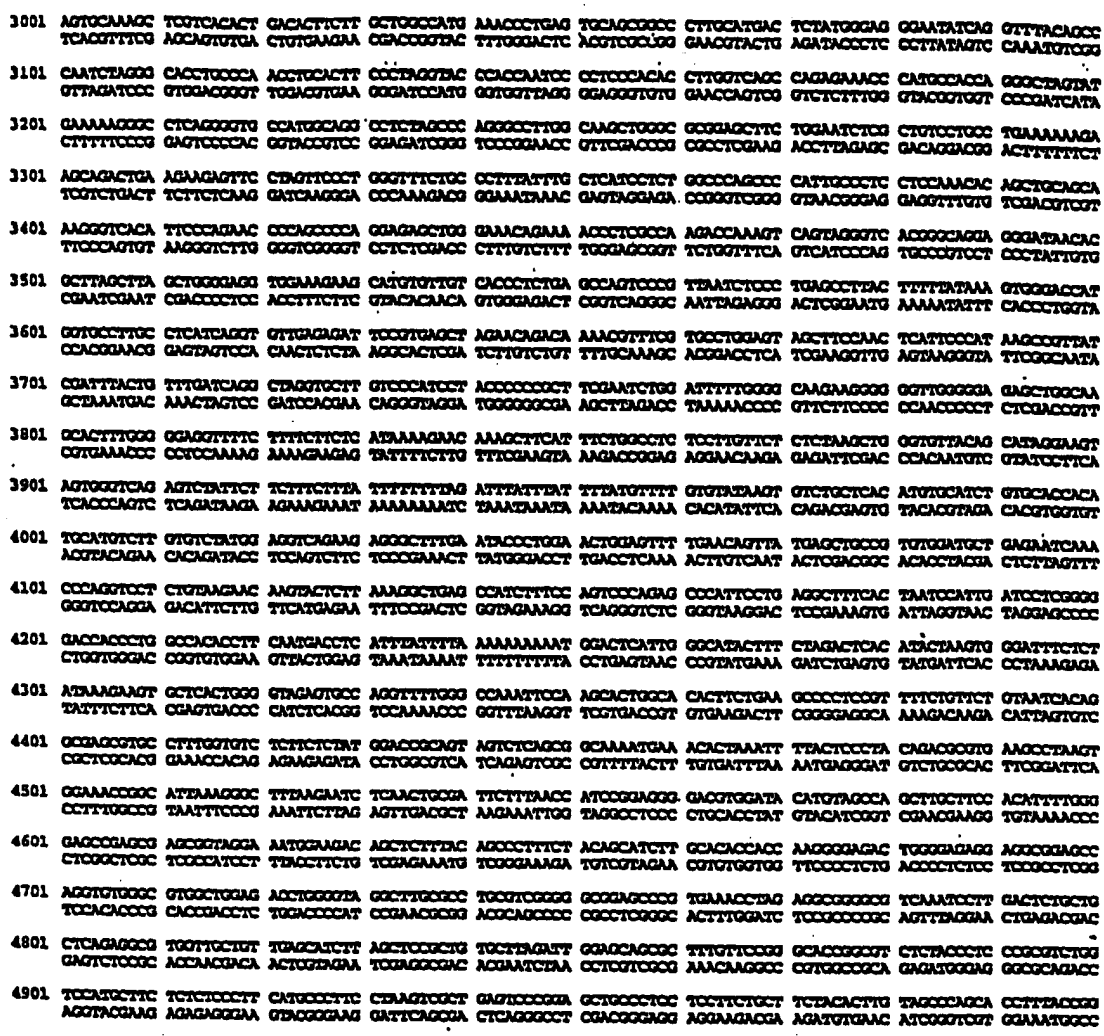
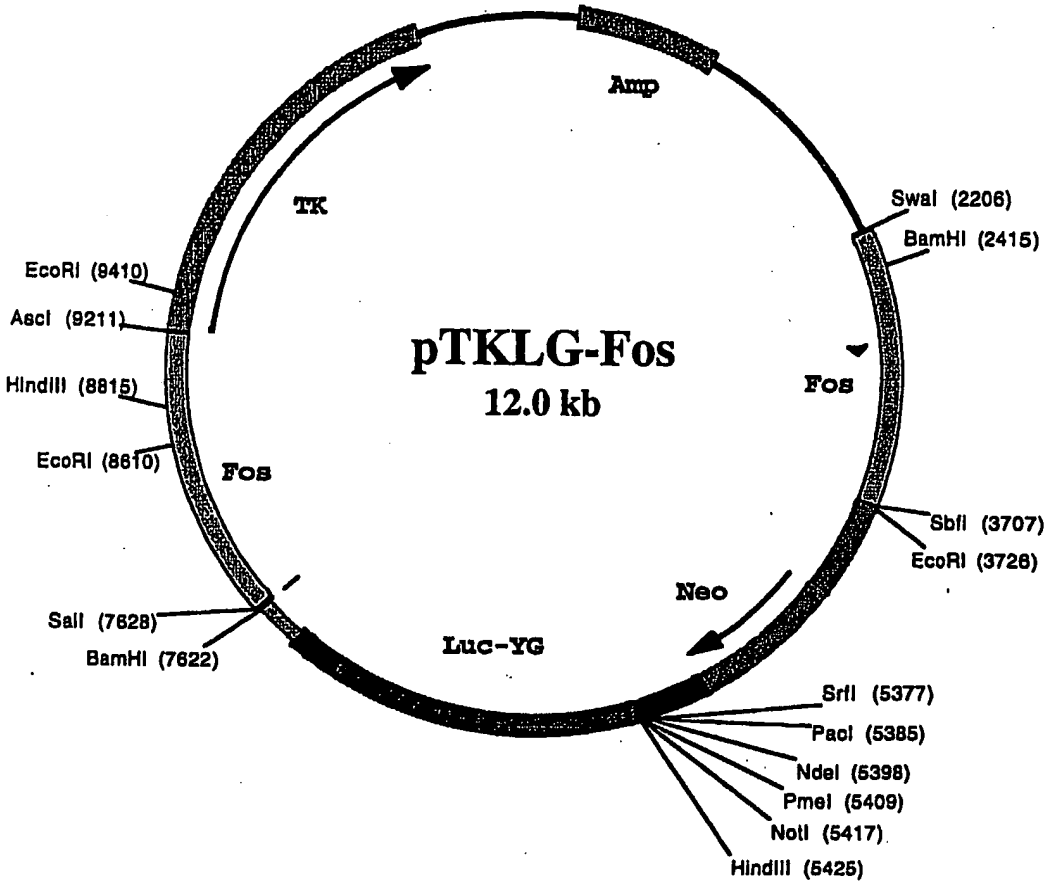


FIG. 3C-2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Fos
Fos
Neo
Luc-YG
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EcoRI (3726)
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PacI (5385)
NdeI (5398)
PmeI (5409)
NotI (5417)
HindIII (5425)
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HindIII (8815)
AscI (9211)
EcoRI (9410)

FIG. 4A

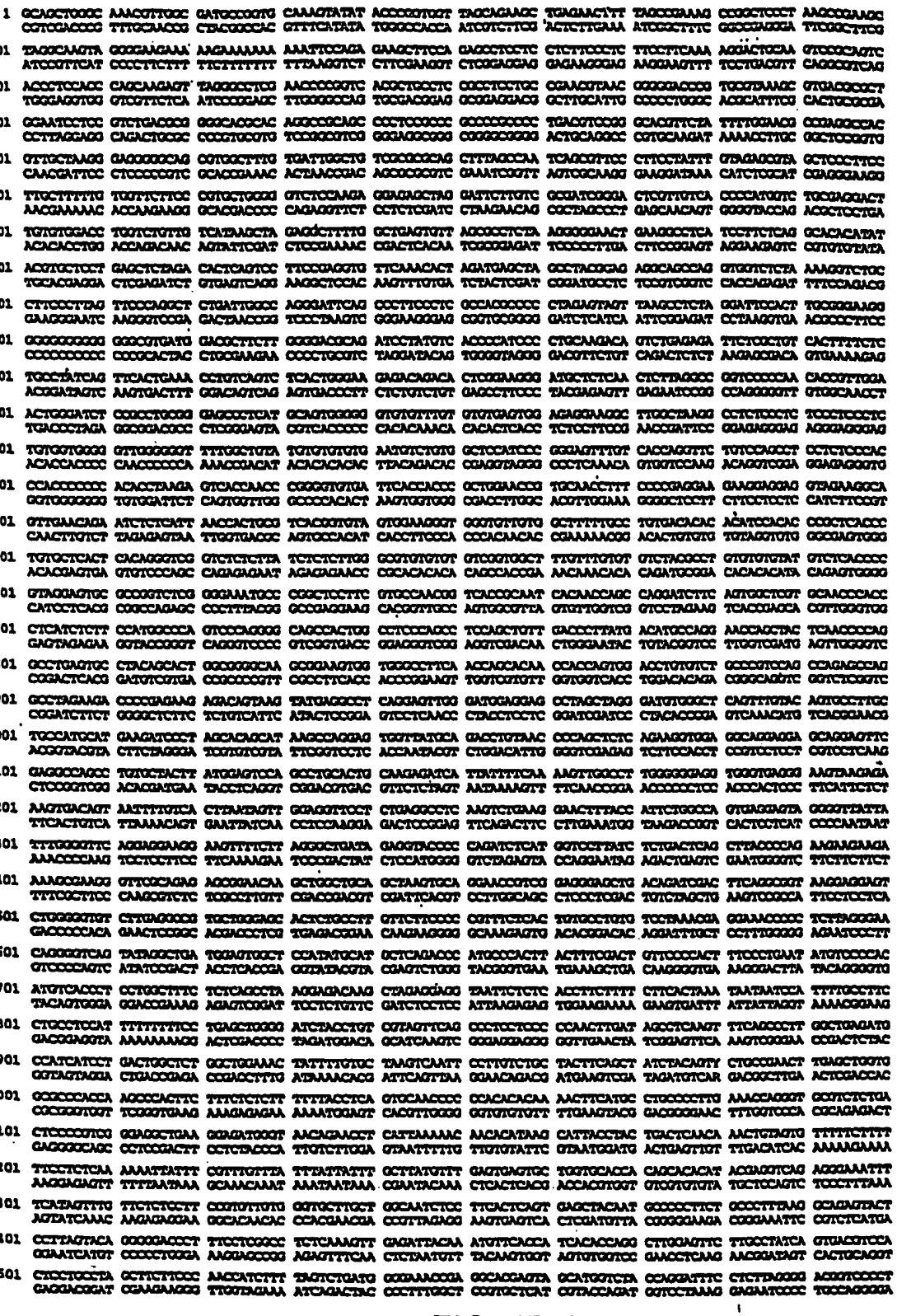

FIG. 4B-1

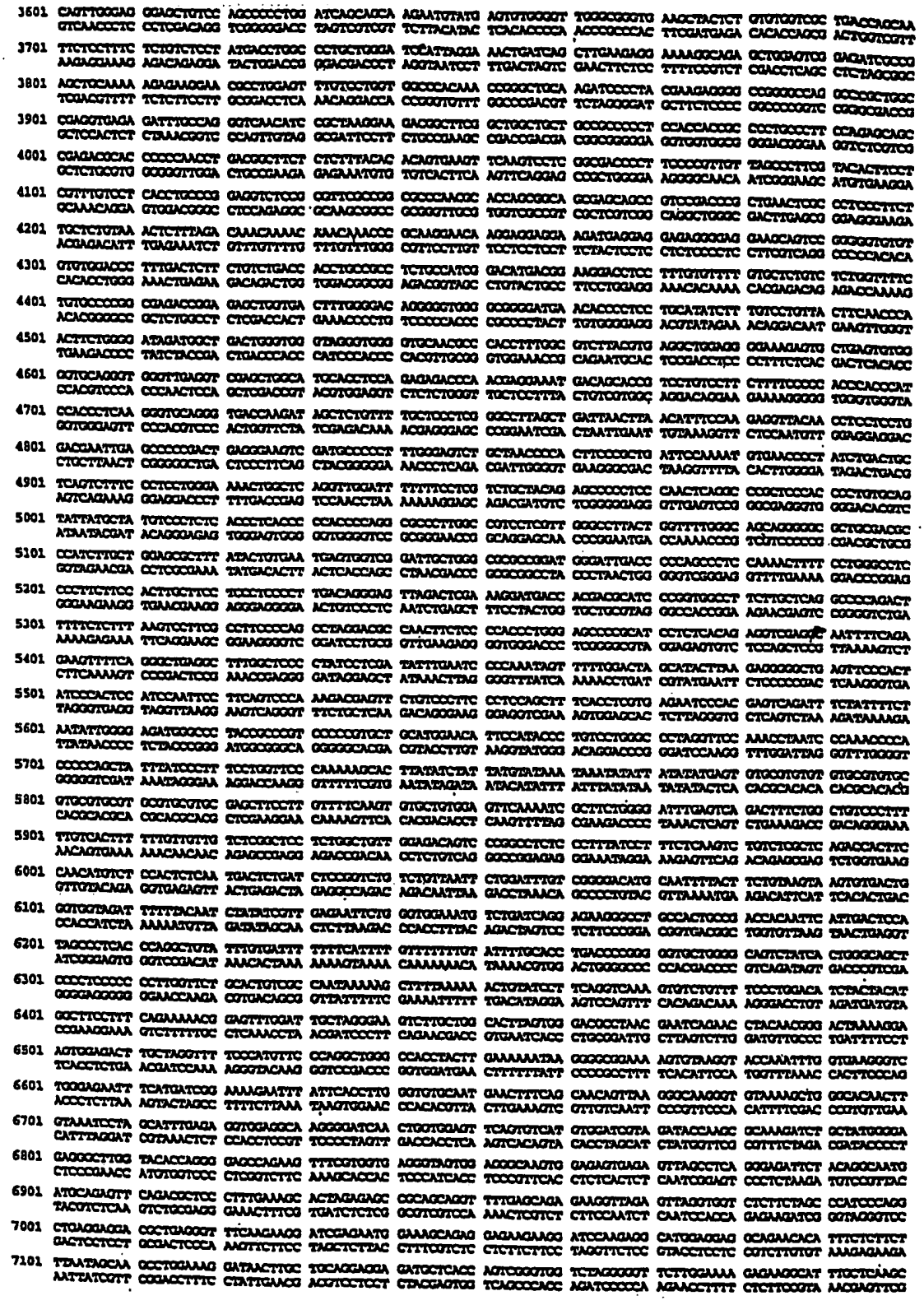

FIG. 4B-2

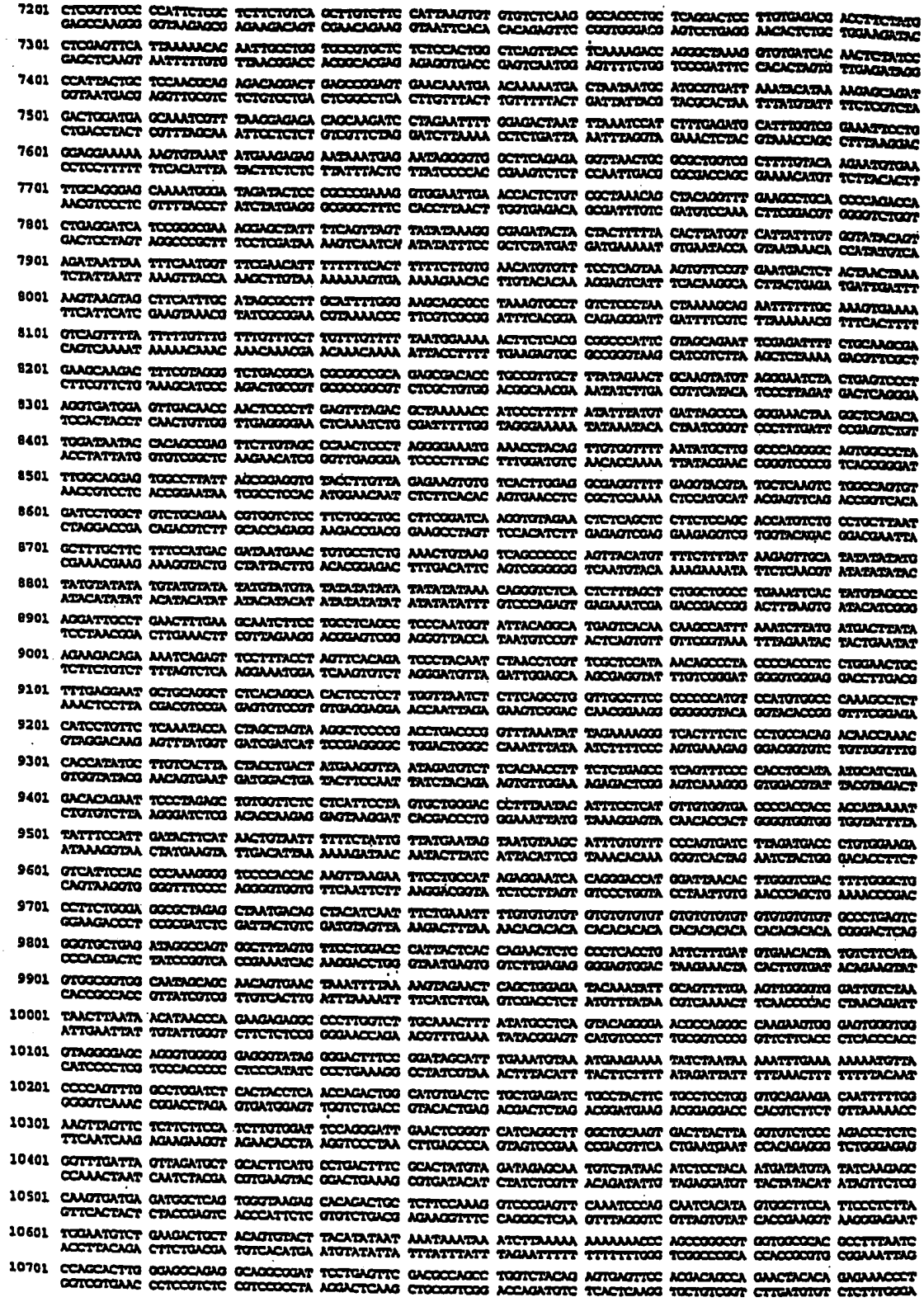

FIG. 4B-3

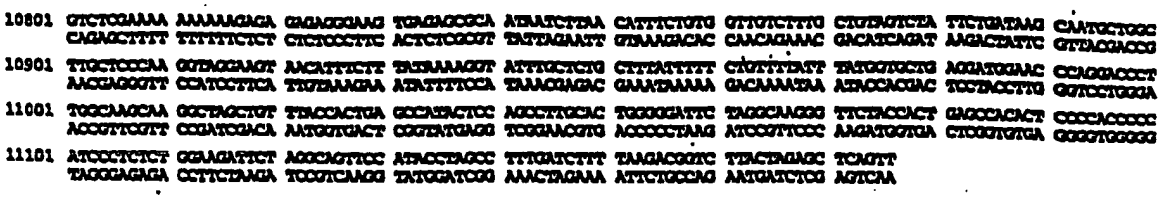

FIG. 4B-4

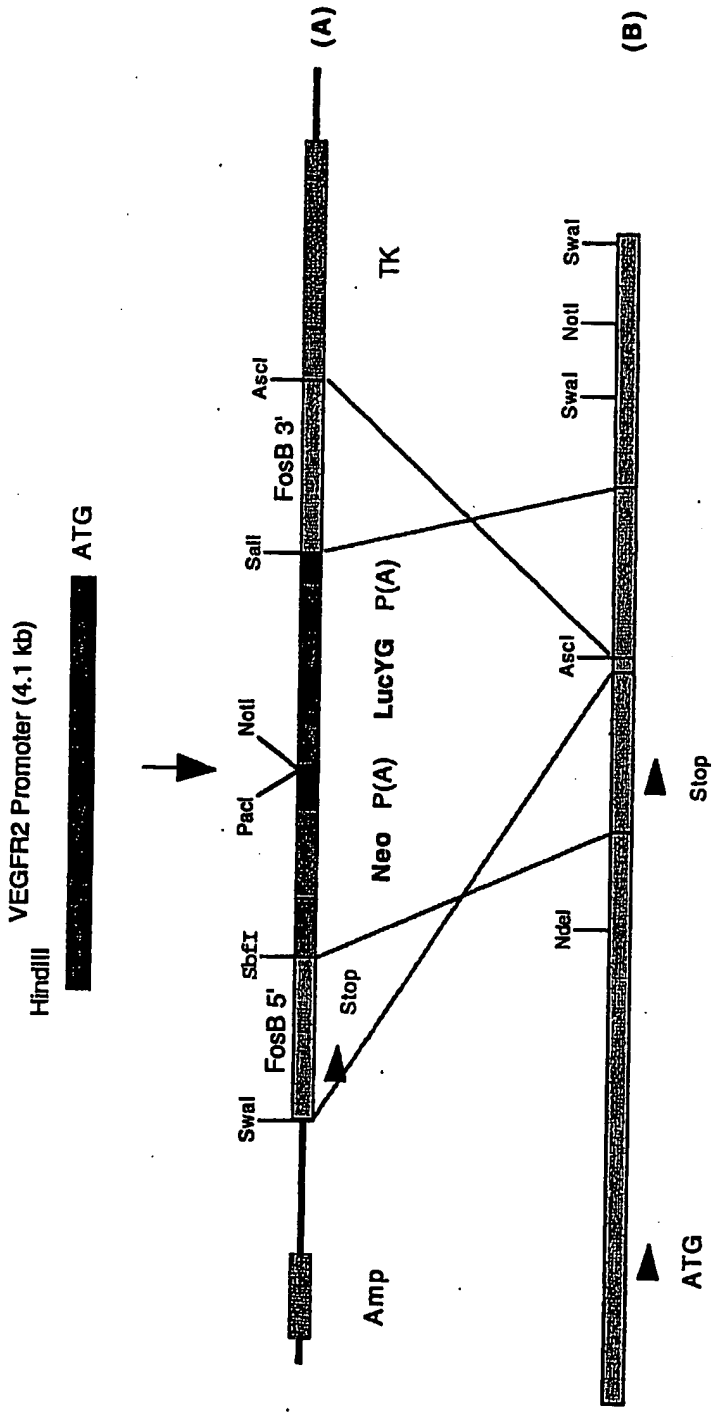
(A)
(B)
TK
AscI
FosB 3'
SalI
NotI
PacI
SbfI
FosB 5'
SwaI
Stop
LucYG
P(A)
Neo
P(A)
Amp
SwaI
NotI
SwaI
AscI
Stop
NdeI
ATG
ATG
HindIII
VEGFR2 Promoter (4.1 kb)

FIG. 5A

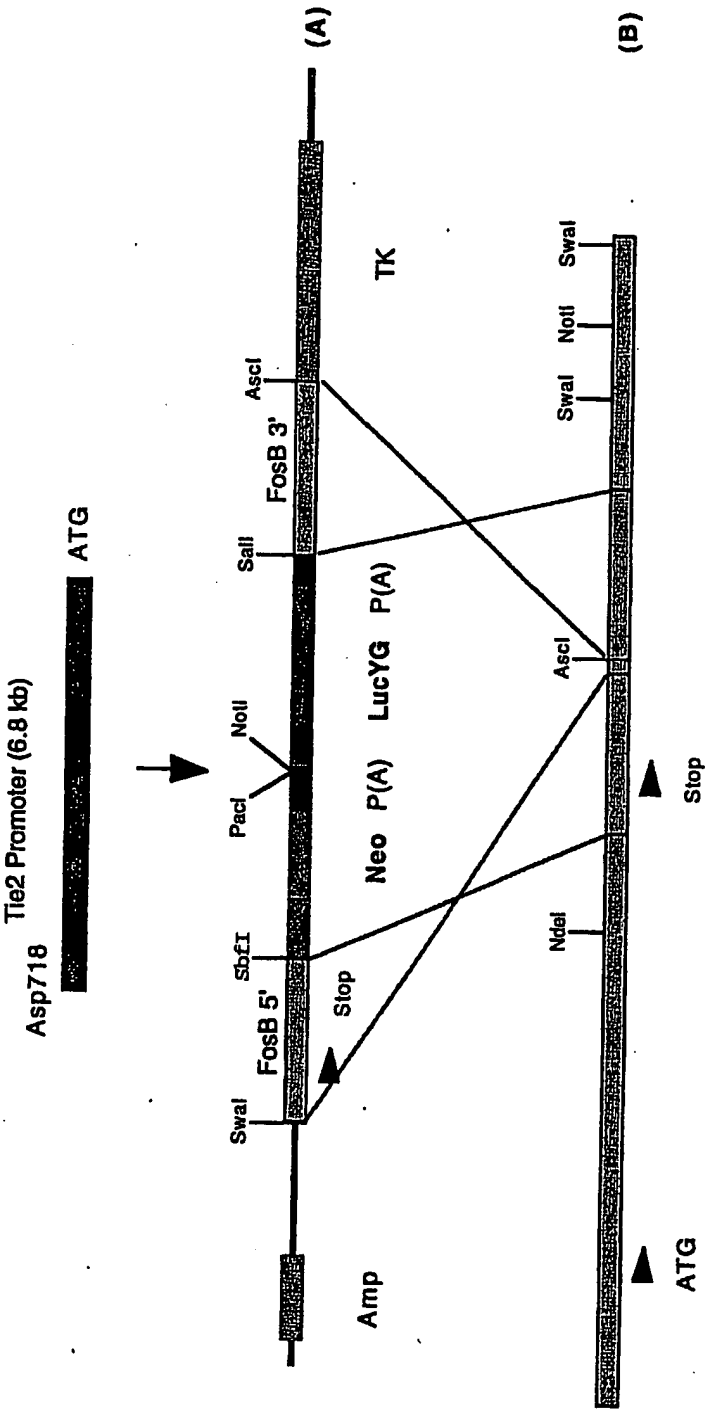
(A)
(B)
TK
SwaI
NotI
SwaI
AscI
FosB 3'
SalI
ATG
Tie2 Promoter (6.8 kb)
Asp718
LucYG P(A)
AscI
NotI
PacI
Neo P(A)
Stop
SbfI
NdeI
FosB 5'
Stop
SwaI
Amp
ATG

FIG. 5B

VEGF project	VEGFR2 project	Tie2 project
Screening primers	**Screening primers**	**Screening primers**
Primers: VF1-VR1A Product size: 0.69Kb	Primers: KF1-KR1 Product size: 0.45Kb	Primers: TF3-TR1 Product size: 0.45Kb
PCR program	**PCR program**	**PCR program**
Hot start	Hot start	Hot start
94°C 40 sec 65°C 1 min 30 sec 72°C 1 min 30 sec	94°C 40 sec 58°C 1 min 30 sec 72°C 1 min 30 sec	94°C 40 sec 58°C 1 min 30 sec 72°C 1 min 30 sec
40 cycles	40 cycles	40 cycles
Confirmation primers	**Confirmation primers**	**Confirmation primers**
Primers: VF2-VR2 Product size: 0.98Kb	Primers: KF2-KR2 Product size: 0.58Kb	Primers: TF2-TR1 Product size: 0.47Kb
PCR program	**PCR program**	**PCR program**
Hot start	Hot start	Hot start
94°C 40 sec 65°C 1 min 30 sec 72°C 1 min 30 sec	94°C 40 sec 65°C 1 min 30 sec 72°C 1 min 30 sec	94°C 40 sec 58°C 1 min 30 sec 72°C 1 min 30 sec
40 cycles	40 cycles	40 cycles

FIG. 6

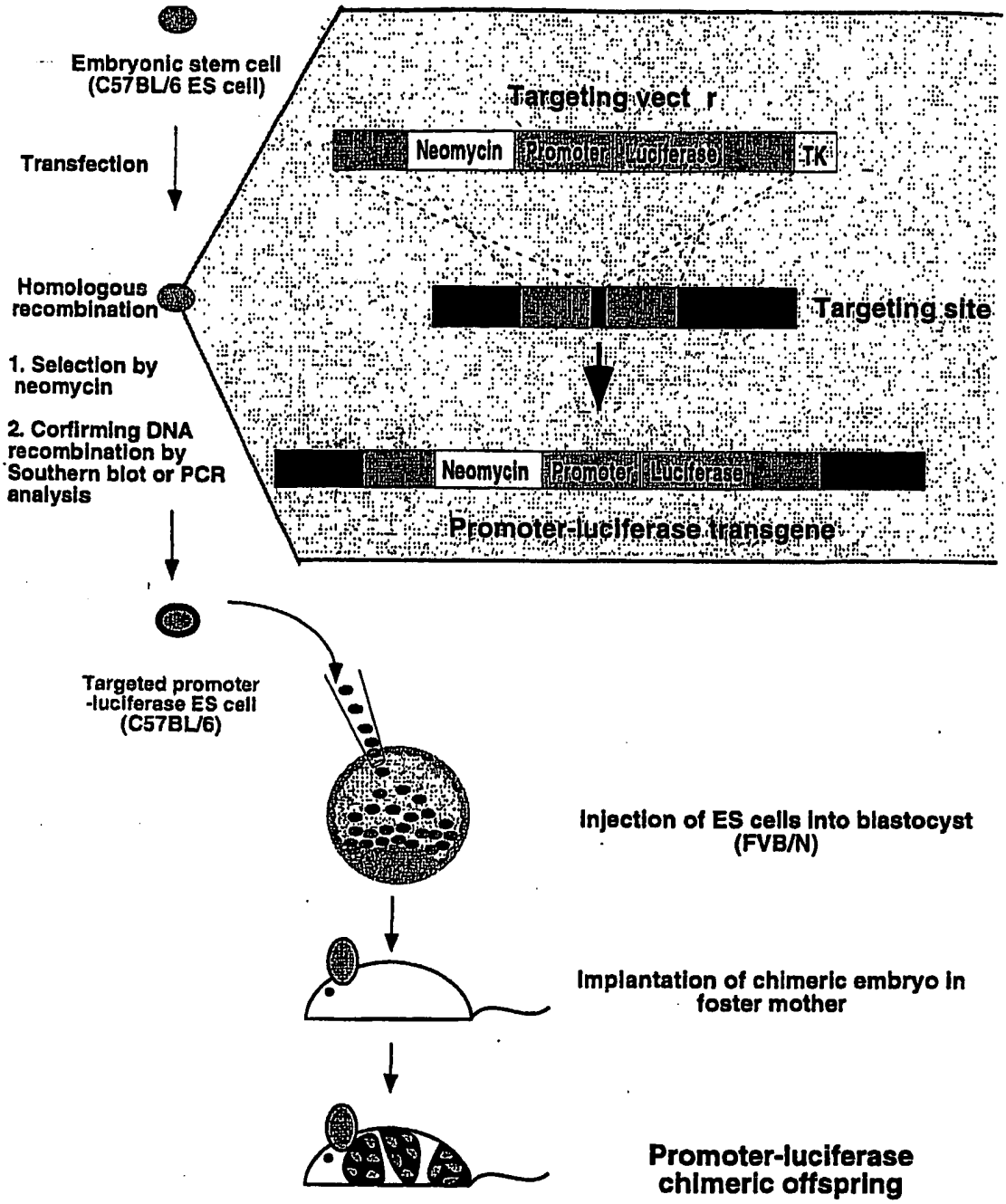
Embryonic stem cell
(C57BL/6 ES cell)
Transfection
Homologous recombination
1. Selection by neomycin
2. Corfirming DNA recombination by Southern blot or PCR analysis
Targeting vect r
Neomycin
Promoter
Luciferase
TK
Targeting site
Neomycin
Promoter
Luciferase
Promoter-luciferase transgene
Targeted promoter -luciferase ES cell (C57BL/6)
Injection of ES cells into blastocyst (FVB/N)
Implantation of chimeric embryo in foster mother
Promoter-luciferase chimeric offspring

FIG.7

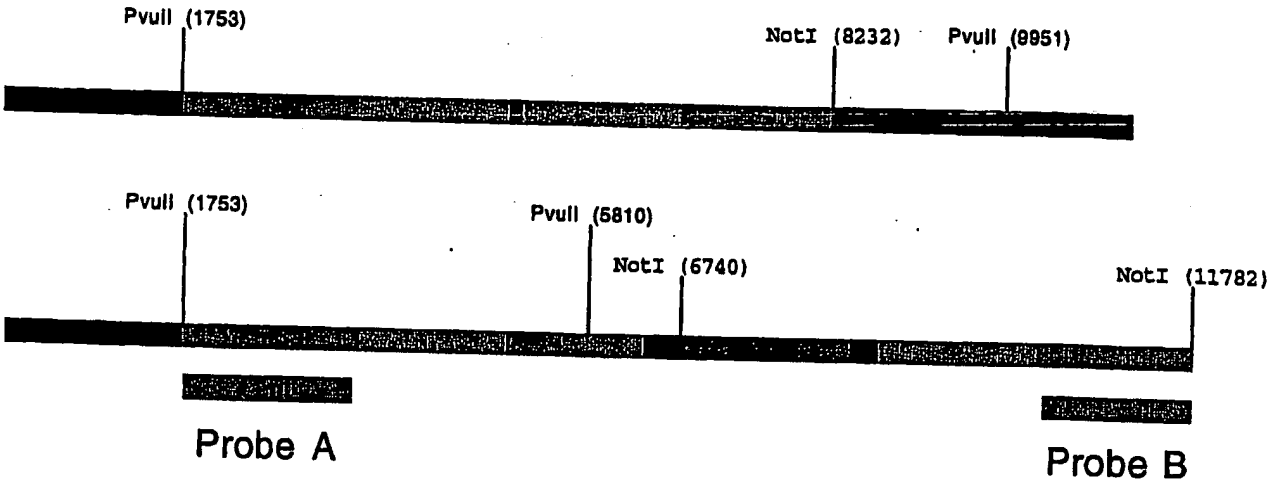
PvuII (1753)
NotI (8232)
PvuII (9951)
PvuII (1753)
PvuII (5810)
NotI (6740)
NotI (11782)
Probe A
Probe B

FIG.8

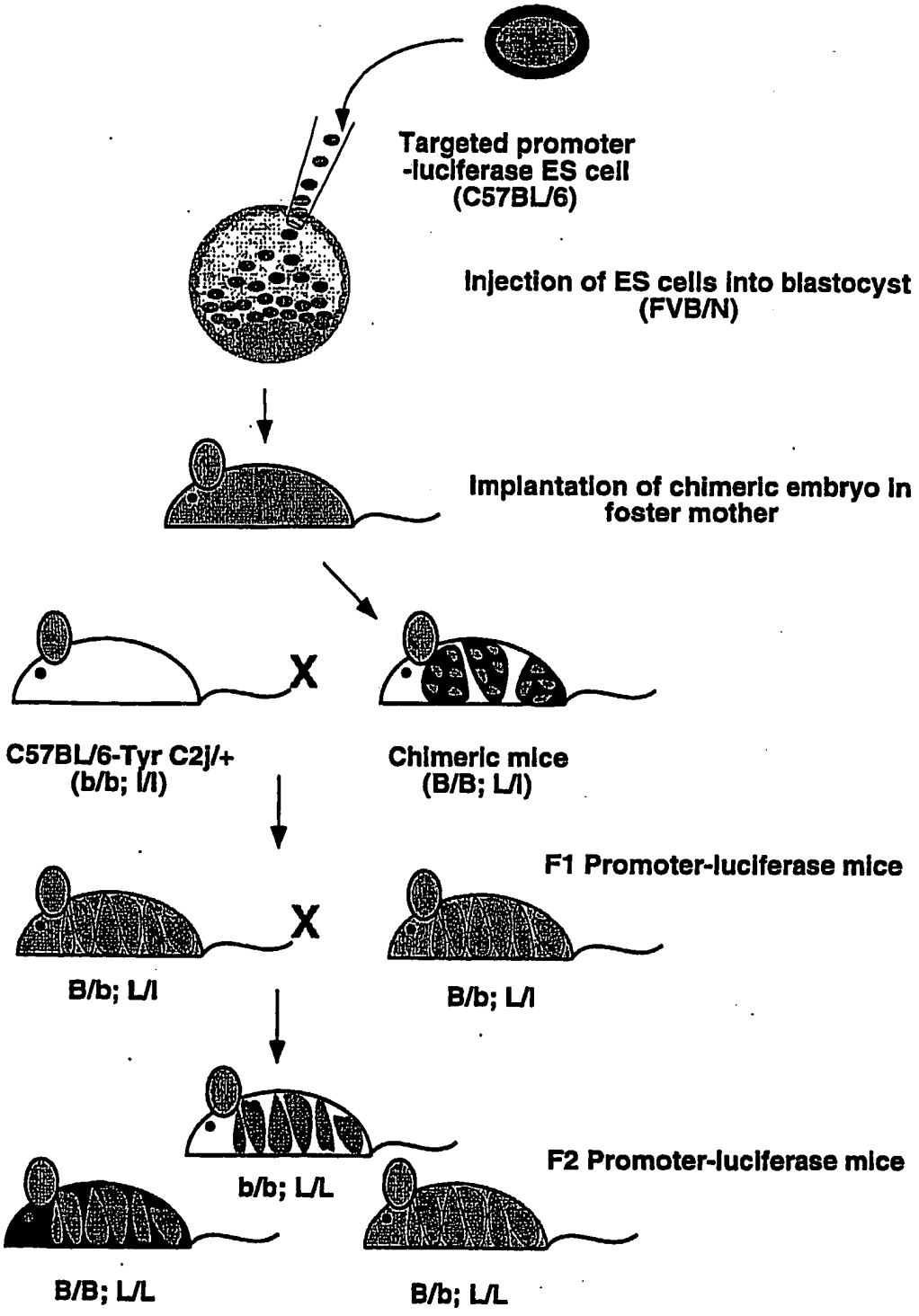
Targeted promoter
-luciferase ES cell
(C57BL/6)
Injection of ES cells into blastocyst
(FVB/N)
Implantation of chimeric embryo in
foster mother
X
C57BL/6-Tyr C2j/+
(b/b; l/l)
Chimeric mice
(B/B; L/l)
F1 Promoter-luciferase mice
X
B/b; L/l
B/b; L/l
b/b; L/L
F2 Promoter-luciferase mice
B/B; L/L
B/b; L/L

FIG.9

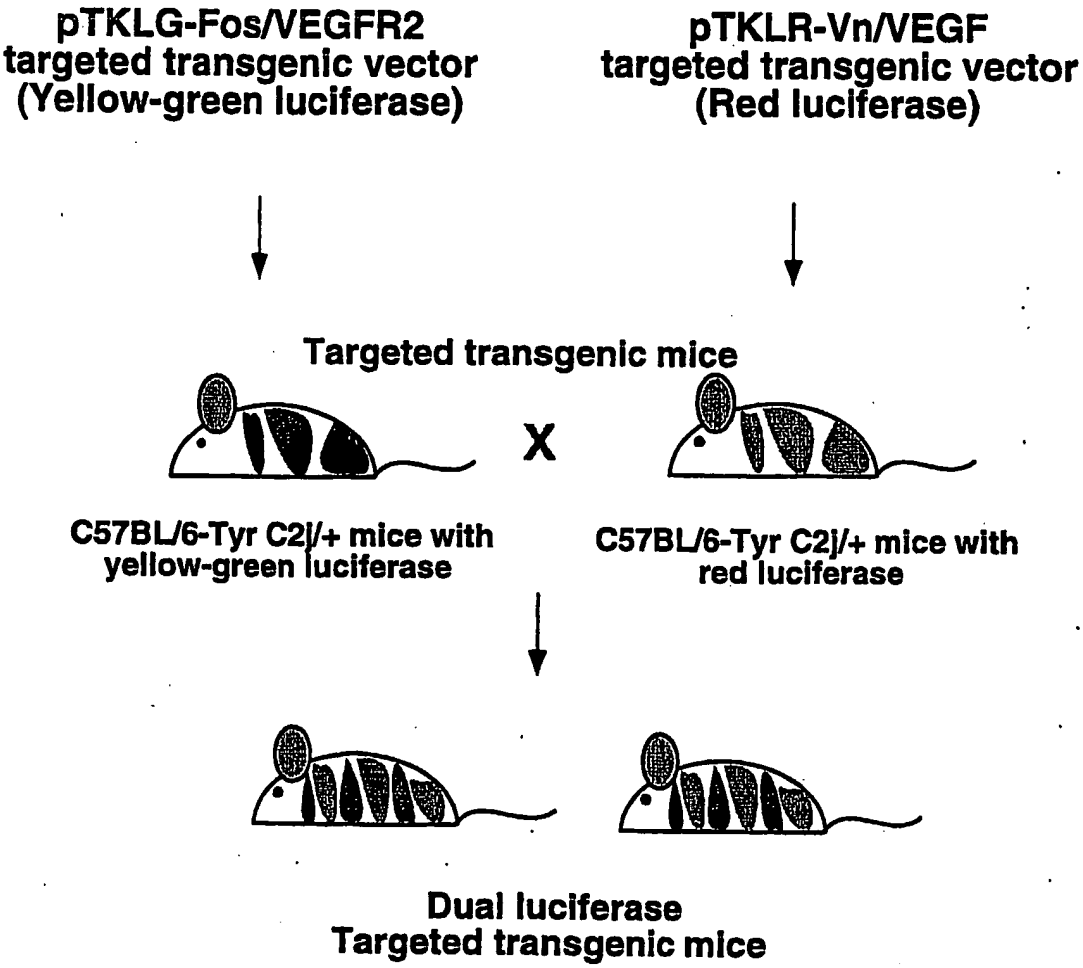
pTKLG-Fos/VEGFR2
targeted transgenic vector
(Yellow-green luciferase)
pTKLR-Vn/VEGF
targeted transgenic vector
(Red luciferase)
Targeted transgenic mice
X
C57BL/6-Tyr C2J/+ mice with
yellow-green luciferase
C57BL/6-Tyr C2J/+ mice with
red luciferase
Dual luciferase
Targeted transgenic mice

FIG.10

SEQUENCE LISTING

<110> XENOGEN CORPORATION

<120> TARGETING CONSTRUCTS AND TRANSGENIC ANIMALS PRODUCED THEREWITH

<130> PXE-008.PC

<140>
<141> 1999-12-16

<150> 60/152,522
<151> 1999-09-03

<160> 39

<170> PatentIn Ver. 2.0

<210> 1
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer PGKF

<400> 1
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<210> 2
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer PGKR

<400> 2
ggctgcaggt cgaaaggccc ggagatgagg 30

<210> 3
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer NeoF

<400> 3
acctgcagcc aatatgggat cggccattga ac 32

<210> 4
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer NeoR

<400> 4
ggatccgcgg ccgcccccag ctggttcttt ccgcctc 37

<210> 5
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer TKF

<400> 5
ggatcctcta gagtcgagca gtgtggtttt 30

<210> 6
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer TKR

<400> 6
gagctcccgt agtcaggttt agttcgtccg 30

<210> 7
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer F5R51

<400> 7
gtacatttaa atcctgcagg 20

<210> 8
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer F5R52

<400> 8
agctcctgca ggatttaaat 20

<210> 9
<211> 77
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer F3R31

<400> 9
ggcccgggct taattaatgc atcatatggt accgtttaaa cgcggccgca agcttgtcga 60
cggcgcgccg gccggcc 77

<210> 10
<211> 77
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer F3R32

<400> 10
gatcggccgg ccggcgcgcc gtcgacaagc ttgcggccgc gtttaaacgg taccatatga 60
tgcattaatt aagcccg 77

<210> 11
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer VN1R

<400> 11
ctgtatttaa atctgcccac cctattcagg acagtagtc 39

<210> 12
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer VN1F

<400> 12
ccaatgcatc aacccagcca ggaggagtgc g 31

<210> 13
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer VN2R

<400> 13
aacgcgtcga cttcggagat gtttcgggga taaccagg 38

<210> 14
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer VN2F

<400> 14
ttggcgcgcc ccatagagaa gagacaccaa aggcacgctc 40

<210> 15
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer FosB1F

<400> 15
ctgtatttaa atcccgtttc tcactgtgcc tgtgtc 36

<210> 16
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer FosB1R

<400> 16
gtctcctgca ggcttcctcc tccttgttcc ttgcg 35

<210> 17
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer FosB2F

<400> 17
aacgcgtcga cggatgggat tgacccccag ccctc 35

<210> 18
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer FosB2R

<400> 18
ttggcgcgcc ccttgcctcc acctctcaaa tgc 33

<210> 19
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer VF1

<400> 19
acctcactct cctgtctccc ctgattccca a 31

<210> 20
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer VR1A

<400> 20
gctctggcgg tcacccccaa aagca 25

<210> 21
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer VF2

<400> 21
ccctttccaa gacccgtgcc atttgagc 28

<210> 22
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer VR2

<400> 22
actttgcccc tgtccctctc tctgttcgc 29

<210> 23
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer KF1

<400> 23
gctgcgtcca gatttgctct cagatgcg 28

<210> 24
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer KR1

<400> 24
ttctcaggca cagactcctt ctccgtccct 30

<210> 25
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer KF2

<400> 25
cagatggacg agaaaacagt agaggcgttg gc 32

<210> 26
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer KR2

<400> 26
gaggactcag ggcagaaaga gagcg 25

<210> 27
<211> 29
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer TF3

<400> 27
agcttagcct gcaagggtgg tcctcatcg 29

<210> 28
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer TF2

<400> 28
caaatgcacc ccagagaaca gcttagcctg c 31

<210> 29
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<220>
<223> Description of Artificial Sequence: primer TR1

<400> 29
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<210> 30
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<220>
<223> Description of Artificial Sequence: primer F51

<400> 30
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<210> 31
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer R51

<400> 31
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<210> 32
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<220>
<223> Description of Artificial Sequence: primer F52

<400> 32
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<210> 33
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<212> DNA
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<220>
<223> Description of Artificial Sequence: primer R52

<400> 33
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<210> 34
<211> 32
<212> DNA
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<220>
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<400> 34
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<210> 35
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer R31

<400> 35
cggctcccca aaatgtggaa gcaagc 26

<210> 36
<211> 30
<212> DNA
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<220>
<223> Description of Artificial Sequence: primer F32

<400> 36
gaatccatct tgctccaaca ccccaacatc 30

<210> 37
<211> 26
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<400> 37
cgcctcctct ccccagtctc cccttg 26

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catagagtca cctcggaagg caaagaggga cccattcttg agatccgtga aggcgtcaaa 2580
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/90 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 30715 A (ISACOFF EHUD Y ;SIEGAL MICAH S (US); UNIV CALIFORNIA (US); CALIFOR) 16 July 1998 (1998-07-16) page 29, line 5 -page 30, line 10; claims	1-8,10, 22-26, 32,34,35
X	WO 98 28971 A (LINK CHRISTOPHER ;UNIV TECHNOLOGY CORP (US)) 9 July 1998 (1998-07-09)	1,2,8, 10,12, 14-17
Y	page 22, line 3 - line 6; figure 1	1,2,7, 10-12, 14,17-20
	page 26, line 24 - line 28; claims 11-19; figure 1; example 3 page 18, line 9 - line 13	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2000	03/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chambonnet, F

C.(Continuation) DOCUMENTS CONSIDERED T BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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WO 9828971 A	09-07-1998	AU 5619298 A	31-07-1998
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